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THE ZINC CONTENT OF SOME FOOD PRODUCTS.

By VICTOR BIRCKNER.

(From the Bureau of Chemistry, Department of Agriculture, Washington.)

(Received for publication, April 2, 1919.)

The fact that zinc is a normal constituent of the ash of many foodstuffs has been largely overlooked. The papers by Lechartier and Bellamy,[1] Mendel and Bradley,[2] Javillier,[3] and Weitzel,[4] while establishing the presence of the "white metal" in various animal and vegetable tissues, did not greatly stimulate work along this line owing to the fact that the methods used by these investigators were too laborious to be applicable for routine determinations. The need for a process by which it is possible to determine amounts of less than 1 mg. of zinc expeditiously and with a fair degree of accuracy, is, therefore, keenly felt. The turbidimetric method recently suggested by Breyer[5] undoubtedly fulfills these requirements, and after a few trials, its use was decided upon for the purpose of this investigation.

The method as used by myself was devised primarily for the determination of zinc in cow's milk. For this reason, the precipitations of the zinc sulfide were made from solutions of the chloride, instead of the sulfate, as usual, because, owing to the preponderance of calcium in the ash of milk, there seemed to be some danger of losing zinc through occlusion in the calcium sulfate from which the sulfate solution would have to be filtered off. The precipitation of zinc sulfide from a chloride solution, and its

[1] Lechartier, G., and Bellamy, F., *Compt. rend. Acad.*, 1877, lxxxiv, 687.

[2] Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiv, 313.

[3] Javillier, M., Thesis, Univ. of Paris, 1908.

[4] Weitzel, A., *Zentr. Physiol.*, 1914, xxviii, 766.

[5] Breyer, F. G., in Scott, W. W., Standard methods of chemical analysis, New York, 2nd edition, 1917, 487.

filtration on the following day, were effected without difficulty in nearly all cases. All ashes were prepared in platinum, using an electric muffle. The details of the process are given below.

Preparation of Standard Zinc Solution.

1.2447 gm. of pure ignited zinc oxide (Kahlbaum) are dissolved in hydrochloric acid in a 1 liter volumetric flask. Ammonia is then added in such amount as to leave the solution clear and but faintly acid. The flask is now filled up to the mark with distilled water and shaken. For the turbidimetric estimation, a 10 cc. portion of this solution is diluted to 100 cc. in a volumetric flask. Each cc. of this solution contains 0.1 mg. of zinc.

Preparation of Ferrocyanide Solution.

34.8 gm. of pure potassium ferrocyanide (Kahlbaum) are dissolved in distilled water, and the solution is made up to 1 liter. The solution may have to be filtered, but need not be renewed for long periods, if kept in a dark place.

Precipitation of Zinc as Sulfide.

A given portion of the material in question is weighed into a platinum dish and carefully ashed at low heat, using an electric muffle. The ash is dissolved in hydrochloric acid and the solution transferred to a so called beaker flask of Kavalier Bohemian glass (the use of borosilicate glass of the Jena type should be avoided on account of the zinc content of these glasses). To the solution in the flask are added a few drops of methyl orange indicator, and then, in portions, dry sodium carbonate until the solution turns yellow. 50 per cent formic acid solution is now added drop by drop until the red color just reappears; then two or three drops are added to prevent precipitation of iron, the amount depending, of course, on the volume of the solution. The flask is now heated to near the boiling point and a lively stream of H_2S gas passed through the liquid until the latter has cooled completely. The flask is covered and allowed to stand over night.

The precipitate is collected on a small filter and washed with hydrogen sulfide water, slightly acidulated with formic acid. A clear filtrate should be obtained.[6]

Turbidimetric Estimation of Potassium Zinc Ferrocyanide.

The method employed differs in several respects from the one recently described by Meldrum[7] for the estimation of zinc in water. The use of ordinary Nessler tubes was soon abandoned, since the estimation of slight differences in the turbidity, even when viewed against a black background, was found to give very uncertain results. By using the so called Nessler jar,[8] a cylindrical vessel with an inside diameter of 32 mm., with lip and polished bottom, and graduated at 50 and 100 cc., the method can be put on an accurate basis. The determinations are made by placing the jar, which is filled up to the 50 cc. mark with the solution to be tested, on a clean sheet of print, preferably a table of figures, *e.g.* a table of logarithms. By looking down through the column of liquid, the print will appear more or less turbid depending on the amount of potassium zinc ferrocyanide held in suspension. If only jars with plane bottoms are used differences in turbidity may be readily seen which it would be quite impossible to distinguish by viewing the vessel from the side. By keeping in readiness a number of jars containing measured volumes of the standard zinc solution and using the same amount of acid and of ferrocyanide for each determination, the zinc content of a given solution can be quickly ascertained by comparison with these standards. In the analyses reported below, the amounts of acid and ferrocyanide contained in the final 50 cc. of solution were 3 cc. of concentrated hydrochloric acid and 5 cc. of the ferrocyanide solution in each instance. For accurate results the analyst will do best to concentrate his attention on one or two simultaneous determi-

[6] In my own work, when investigating a new substance, I have always made a preliminary precipitation with hydrogen sulfide from an acid solution (1 part of concentrated HCl in 20 parts of solution) using a pressure bottle. But since there was nothing removed thereby in any case which would have interfered with the turbidimetric determination of zinc as described below, the first H_2S treatment is probably unnecessary.

[7] Meldrum, R., *Chem. News*, 1917, cxvi, 295, 308.

[8] See Eimer and Amend Catalogue C, 1913, No. 4152.

nations, especially since the whole procedure requires but a few minutes. The following are the details of the process.

The small filter holding the zinc sulfide is treated with 6 cc. of a hot solution of hydrochloric acid (1:1), the filtrate being poured back once to be finally collected in a Nessler jar. The filter is washed with distilled water until the liquid in the jar stands somewhat below the 50 cc. mark. Definite volumes of the standard zinc chloride solution are measured from a burette into three or four Nessler jars, into each of which water and 3 cc. of concentrated hydrochloric acid are also placed. The strength of the standard set of solutions is chosen so as to come near the amount of zinc expected in the sample. Thus, if 0.3 mg. is expected, the standard set may contain 2.5 cc., 3.0 cc., and 3.5 cc. of the standard zinc solution respectively in each jar. When all the jars are in readiness, 5 cc. of the potassium ferrocyanide solution are pipetted quickly into each, the liquid is brought to the 50 cc. mark with distilled water, shaken, and the jars are at once placed on the printed sheet for comparison.

Sources of Error, Sensitiveness, and Limitations of the Method.

Sources of error which should be guarded against are a loss of zinc during ignition and an incomplete precipitation of the zinc sulfide due to an excessive concentration of formic acid. Both these errors would cause low results. In general, provided no zinc is introduced with the reagents or from the glassware, there is little chance for obtaining high results with this method. With substances from which zinc is lost through reduction during the ignition, a strong acid digestion or a careful ashing with the addition of free sulfuric acid may be resorted to.

The turbidimetric method, as outlined, can be used directly only in cases where the amount of metallic zinc in the sample is below 0.5 mg. With larger amounts, the turbidity produced becomes so strong as to render invisible the printed characters on the sheet below the column of liquid. In such cases, provided the zinc content of the sample is still below 1 mg., it may be determined accurately by adding another 5 cc. of ferrocyanide solution, 3 cc. of hydrochloric acid, then diluting the contents of the jar to the 100 cc. mark with distilled water, mixing by pouring

into a second Nessler jar, and finally dividing the liquid equally between the two jars, with either one of which the reading may be obtained. If the zinc sulfide precipitate is heavy, it is necessary to collect the dissolved precipitate in a volumetric flask, and to use an aliquot portion for the determination.

The amount of zinc necessary to respond to the turbidimetric test is small, the complex potassium zinc ferrocyanide being highly insoluble. With solutions containing as little as 0.02 mg. of metallic zinc a perceptible turbidity develops within a few moments after adding the ferrocyanide solution.

The mixtures in the jars gradually turn more turbid on standing. For this reason the final mixture of the sample and the standard solution with which it is to be compared should be made up as nearly simultaneously as possible. With a solution of unknown zinc content, this may be done as follows: A series of Nessler jars, each holding 3 cc. of concentrated hydrochloric acid, a measured volume of the standard zinc solution, and water, are prepared. The first 5 cc. of ferrocyanide solution are now run into the solution to be tested. From the degree of turbidity which develops, the experienced analyst can judge at once the approximate zinc content of the solution. The second 5 cc. of the ferrocyanide solution are then quickly pipetted into a jar of the standard set containing somewhat less zinc than there appears to be in the sample. The final adjustment is made by comparing the two jars on a sheet of print and adding small amounts of standard zinc solution from the burette to the jar from the standard set until the turbidity of the latter equals that of the sample.

For the purpose of testing the accuracy of the method, some determinations were made on samples of skimmed milk to which measured amounts of standard zinc solution had been added. The results in Table I show that the method is sufficiently accurate for all practical purposes. If properly carried out, the experimental error should not be over ± 0.05 mg.

The determinations in Table II were made in order to ascertain what error in the results might be caused by the handling and washing of very small amounts of zinc sulfide precipitate in the average determination. Measured volumes of standard zinc solution were placed into flasks and diluted with water by an-

other chemist. The samples were then handed to me for analysis by precipitation as sulfide and subsequent turbidimetric determination. The results given in Table II show that no appreciable loss of zinc need occur during the filtration of the sulfide, the amount of zinc recovered being well within the ±0.05 mg. limit which is to be allowed for the method in general.

TABLE I.

Analysis of Skimmed Milk Samples to Which Small Quantities of Zinc Chloride Solution Had Been Added.

Amount of sample.	Zinc content of sample.	Amount of zinc added.	Total zinc present.	Zinc found.
cc.	*mg.*	*mg.*	*mg.*	*mg.*
25	0.11	0.23	0.34	0.31
25	0.11	0.39	0.50	0.45
50	0.22	0.17	0.39	0.40
75	0.34	—	—	—

TABLE II.

Loss of Zinc Due to Handling and Washing of Zinc Sulfide Precipitate.

Sample No.	Zinc in sample.	Zinc found.
	mg.	*mg.*
1	0.15	0.13
2	0.05	0.04
3	0.11	0.09
4	0.15	0.18
5	0.20	0.18
6	0.30	0.26
7	0.20	0.17

Application of Method.

The method as outlined above was at first applied to samples of cow's milk; later a number of other food materials were included in the investigation. Zinc was found in nearly all of them, as shown by the results in Table III.

DISCUSSION.

The data contained in Table III are noteworthy in two respects. They illustrate not only the wide distribution of the element zinc in animal and vegetable products, but they also point

strongly to a certain nutritive function possessed by this element, the exact nature of which is not at present understood. Since the food as well as the bodies of men and animals normally contain small amounts of zinc, special feeding experiments with a zinc-free diet very likely would throw light on this question.

In the brief abstract of his work, Weitzel[4] reports the constant presence of zinc in the urine and the feces of men. Working for over a month on three individuals, he found that from 2.7 to 12.8 mg. of zinc were eliminated daily with the feces. From the results of this author and those of Mendel and Bradley[2] it would seem that zinc is stored especially in the liver of animals, and the latter authors suggest that it might exert some influence on the respiratory functions of the blood. The animal used in their experiments was the mollusk *Sycotypus canaliculatus*, in the dry matter of which they found 1.7 per cent of zinc. They suggest, moreover, that all marine animals may contain this element in their tissues since sea water itself, according to Dieulafait,[9] contains at least 2 mg. of zinc per cubic meter. From my own results in Table III it appears that oysters and agar-agar, both of which are products of the ocean, actually contain notable amounts of this metal.

Weitzel does not describe his method of investigation aside from saying that the zinc was weighed as oxide (presumably after ignition of the sulfide). As appears from his table, it was necessary to ash relatively large amounts of material, which constitutes a serious drawback to his method. His findings are nevertheless of great interest. He evidently was the first to make a quantitative determination of zinc in cow's milk and in eggs. My own findings give higher zinc values for these two products, but the differences between my results and those of Weitzel are not marked. For eggs, my own studies have revealed the fact that practically all the zinc in a hen's egg is contained in the yolk. This finding alone points strongly to the physiological importance of the element for the development of the young.

The zinc content of cow's milk was at first studied on samples of mixed market milk which is sold in bottles by several dealers in the District of Columbia. The zinc content of these samples, as

[9] Dieulafait, L., *Compt. rend. Acad.*, 1880, xc, 1573.

TABLE III.

Zinc Content of Various Food Products.

	Amount of sample.	Amount of ash found.	Zinc (Zn.).		
			Found in sample.	In 1,000 gm. of fresh substance.	Calculated as per cent of ash.
	gm.	*per cent*	*mg.*	*mg.*	*per cent*
Tap water, Washington, D. C.	500.00 cc.	0.0067	0.125	0.25	0.38
Yolk of egg (mixture of 3 yolks)	16.6234	1.39	0.74	44.50	0.32
White of egg	39.1229	—	Trace.	—	—
Egg shell	6.4825	—	0.0	0.0	0.0
Yolk of egg	17.6330	1.52	1.00	56.71	0.37
Whole egg (addition of above 3 parts)	63.4430	—	1.00	15.76	—
Soy bean, dry	0.8421	3.62	0.10	0.12	0.33
Smooth pea, dry	4.9225	3.2 (sulfate ash.)	0.17	34.53	0.11
Carrot	26.1800	0.82	0.13	4.96	0.06
Bone ash	0.3598	—	0.14	—	0.01
Baker's yeast (8.07 per cent moisture)	2.6515	7.65	1.10	414.86	0.54
Barley malt	1.8190	3.02	0.20	11.00	0.36
Barley, (Chevalier, Pacific Coast)	2.6225	2.34	0.07	26.70	0.11
Corn (*Zea mays*), whole kernel	3.9620	1.09	0.10	25.24	0.23
" " " kernels degerminated	5.0146	0.30	0.02	3.99	0.13
Wheat bran	2.2980	4.36	0.32	139.20	0.32
Wheat I, (Dietz check, Va.)	4.0088	1.78	0.34	84.80	0.48
" II, (Fultz, Va.)	4.0255	1.67	0.18	44.71	0.27
" III, (Early Baart, Pacific Coast)	4.6020	2.11	0.12	26.08	0.12
" IV, (Marquis, S. Dakota)	4.2201	2.22	0.20	47.40	0.21

Oat I, (Red oats, Cal.)	4.0995	3.48	0.13	31.71	0.09
" II, (Abundance, S. Dakota)	3.8610	2.79	0.125	32.38	0.12
" III, (Winter Turf, Va.)	2.0254	3.13	0.10	49.37	0.16
" IV, (Canadian, Idaho)	4.4073	3.66	0.17	38.57	0.10
Rye	5.2362	1.60	0.09	17.19	0.10
Rice, unhulled, Japanese	4.0956	5.93	0.06	14.65	0.03
Shred agar (Merck and Co.)	5.8336	5.04	0.08	13.71	0.03
Gelatin (A. H. Thomas Co., No. 33)	4.3799	2.13	0.12	27.40	0.13
Shucked oyster	14.6907	1.85	17.00	1,157.25	6.20
Skimmed milk	50.00 cc.	—	0.22	4.40	—
Mixed market milk:					
No. 1	150.00	—	0.60	4.00	—
" 2	100.00	—	0.43	4.30	—
" 3	100.00	—	0.56	5.60	—
" 4	83.00	—	0.40	5.00	—
" 5	83.00	—	0.35	4.20	—
" 6	83.00	—	0.32	3.90	—
" 7	83.00	—	0.31	3.80	—
" 8	83.00	—	0.31	3.80	—
" 9	83.00	—	0.35	4.20	—
" 10	83.00	—	0.33	4.00	—
" 11	83.00	—	0.32	3.90	—
" 12	83.00	—	0.30	3.60	—
Average of 12 samples		0.7 (estimated.)		4.20	0.06 (estimated.)

TABLE III—*Concluded.*

	Amount of sample.	Amount of ash found.	Zinc (Zn.).		
			Found in sample.	In 1,000 gm. of fresh substance.	Calculated as per cent of ash.
	gm.	*per cent*	*mg.*	*mg.*	*per cent*
Milk from individual cows:					
Cow 1. 13th day after birth of 1st calf	83.00	—	0.45	5.40	
" 2. 14th " " " " " "	83.00	—	0.44	5.30	
" 3. 23rd " " " " " "	83.00	—	0.37	4.50	
" 4. 23rd " " " " " "	83.00	—	0.29	3.50	
" 5. 204th " " " " " "	83.00	—	0.37	4.50	
" 6. 240th " " " " " "	83.00	—	0.32	4.00	
" 7. 296th " " " " " "	83.00	—	0.41	5.00	
" 8a. 7th " " " " 2nd "	83.00	—	0.44	5.40	
" 8b. 23rd " " " " " "	83.00	—	0.38	4.60	
" 9. Nearly dry.....	83.00	—	0.16	2.00	
" 10. Miscellaneous.....	83.00	—	0.40	4.90	
" 11. "	83.00	—	0.35	4.20	
" 12. "	83.00	—	0.30	3.60	
" 13. "	83.00	—	0.35	4.20	
" 14. "	83.00	—	0.275	3.30	
" 15. "	83.00	—	0.35	4.20	
Neutralization ppt. of cow's milk.					
No. 1.....	0.5932	60.35	0.40	674.31	0.11
" 2.....	0.6082	46.45	0.45	739.89	0.16
Human milk (negro):					
No. 1. 4th day after parturition.....	51.26	—	0.29	5.66	—
" 2. 9th " " "	71.40	0.25	0.82	11.50	0.46
" 3. 15th " " "	67.48	—	0.93	13.78	—

shown in Table III, ranged from 3.6 to 5.6 mg. per kilo, the average of twelve determinations being 4.2 mg. per kilo. The zinc content of the milk from individual cows was then determined. The samples were obtained from the St. Elizabeth Hospital Dairy in the District of Columbia. The cows were milked directly into Kavalier beaker flasks of zinc-free Bohemian glass; the analysis was begun upon reaching the laboratory. From the results obtained it appears that the zinc content of cow's milk varies somewhat in different animals and at different stages of the lactation period. It is evidently highest, about 5.5 mg. per liter, shortly after parturition, then for a long time remains constant at about 4.0 to 4.5 mg. per liter, to drop off markedly near the end of lactation. The first drop is illustrated by the results obtained with Cow 8 (see Table III). On the 7th day after parturition the milk of this cow contained 5.4 mg. of zinc per kilo, while 2 weeks later the value had fallen to 4.6 mg. per kilo.

Interesting results were obtained with samples of human milk, which were secured at the Freedmen's Hospital, Washington, D.C., from three mothers. A given volume of human milk evidently contains considerably more zinc than an equal volume of cow's milk; and it would appear that the zinc content of human colostrum is lower than that of later milk. These findings are of special interest in view of the fact that the total ash content of human milk is only about one-third as high as the ash content of cow's milk. From this and other evidence the conclusion may safely be drawn that zinc, like iron, is not present in milk as a simple inorganic salt, but that it enters into the composition of some organic milk constituent.

Finally I investigated the zinc content of the so called "neutralization precipitate" of cow's milk, which has been the subject of study by Osborne and Wakeman[10] and by Palmer.[11] The neutralization precipitate was prepared in one instance from skimmed milk in the manner described by Palmer. The finely powdered substance when analyzed contained 7.62 per cent moisture and yielded 60.35 per cent ash. Its zinc content as shown in Table III is 674.31 mg. of zinc per kilo. With this preparation a confirmatory test was also made to be sure that the element in

[10] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1916-17, xxviii, 1.
[11] Palmer, L. S., *J. Assn. Offic. Agric. Chemists*, 1916, ii, 4.

question is actually zinc. A portion of the sulfide precipitate was carefully ignited. The resulting oxide was yellow while hot and white after cooling; this behavior is characteristic of zinc. In a second case, the neutralization precipitate was obtained by neutralizing the milk from one cow with ammonia, after removing the casein and albumin in the usual manner. 11.2345 kilos of whole milk yielded 65 gm. of neutralization precipitate (air-dry). The zinc determination on the latter gave 739.89 mg. of zinc per 1,000 gm. of substance, as shown in Table III. Hence the 65 gm., representing 11.2345 kilos of milk, contain 48.09 mg. of zinc, which is equivalent to 4.28 mg. of zinc per 1,000 gm., the normal value of milk. It is therefore evident that if the acidified serum of cow's milk is neutralized, practically all the zinc which the milk contains will come down with the "neutralization precipitate."

The normal occurrence of the element zinc in such vitally important animal products as milk and eggs, as well as in many other foods, deserves the close attention of the physiologist and the student of nutrition; especially since the quantities found are not at all negligible. The iron content of milk, which in spite of more than a century's research cannot be said to be definitely known, is probably not much, if at all in excess of the zinc content; and manganese has been shown by Nockmann[12] to be present in much smaller quantities. Moreover, it has been demonstrated that the amounts of these elements in milk are stationary, and that their quantity in the milk will not increase beyond a certain narrow limit upon increasing their proportion in the food of the animal. The same may be expected to hold for zinc in consequence of the well known selective function of the mammary gland. At any rate, it seems proper to consider the element zinc no longer as an unusual and unnecessary constituent of some few organisms, but as a regular and probably essential ingredient of living protoplasm.

Of the two cereals, wheat and oats, four different varieties were investigated of each. The zinc content of Wheat I is shown in Table III to be rather high. The low zinc content of the Washington tap water also deserves mention. In Weitzel's locality the zinc content of the water was more than nine times as high.

[12] Nockmann, E., Molkereizeitung, Hildesheim, 1914, xxviii, 419.

SUMMARY.

1. Zinc has been found and determined quantitatively in a number of food materials by using the turbidimetric ferrocyanide method.

2. In hen's eggs practically all the zinc is contained in the yolk, amounting to about 0.005 per cent of the latter. White of egg contains a mere trace of the metal at the most. The total amount present is about 1 mg. per egg.

3. The zinc content of ordinary market milk averages about 4.2 mg. of metallic zinc per kilo of milk.

4. The zinc content of the milk from individual cows varies somewhat in different animals. It is highest during the early part of the lactation period.

5. If the acidified serum of cow's milk is neutralized, practically all the zinc which the milk contains will be found in the "neutralization precipitate."

6. The zinc content of human milk is markedly higher than that of cow's milk in spite of the higher total ash content of the latter.

7. From its constant occurrence in the yolk of eggs as well as in cow's and human milk, it is inferred that the element zinc exerts an important nutritive function, the nature of which is not at present understood.

ZINC IN OYSTERS.

By R. S. HILTNER and H. J. WICHMANN.

(*From the Denver Food and Drug Inspection Station, Bureau of Chemistry, United States Department of Agriculture.*)

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In 1904 Bradley[1] reported the presence of zinc and also of copper in the liver of *Sycotypus canaliculatus*, a large carnivorous snail abundant through Long Island Sound, and in *Fulgur carica*, an allied mollusk. Several other forms of marine mollusks from the Sound, including the oyster, *Ostrea virginiana*, were examined with negative or doubtful results in all cases. Later Mendel and Bradley[2] found that in *Sycotypus* the blood and liver tissue alone contain zinc and copper. Mendel and Bradley[2] also observed that zinc was present in the common drill, *Urosalpinx cinerea*, but were unable to find appreciable amounts in the mussels, *Mytilus edulis* and *Modiola pheatula*, in "bloody clams," *Argina pexata*, in hermit crabs, *Eupagurus pollicaris*, in oysters, *Ostrea virginiana*, and in the common crab, *Cancer irroratus*. They clearly stated their belief, however, that other marine forms would probably be found to contain zinc, derived either directly from the sea water or, more probably, in accord with other familiar examples, picked out and retained by some simple organisms or plants, and thence reaching the carnivorous *Sycotypus* through a series of more complex and larger forms, such as the oyster, on which *Sycotypus* feeds. Moreover, ocean water is known to contain slight traces of the less common elements such as arsenic, copper, lead, and zinc. Dieulafait[3] found that sea water contains about 2 mg. of zinc per cubic meter. Copper is also known to be present in sea water and in many marine animals. In 1908 Willard[4] made a study of the copper content of oysters obtained from various districts on the Atlantic coast. He determined the copper by electrolytic deposition.

The results of an analytical search for metals in Tortugas marine organisms were reported by Phillips in 1917.[5] Large amounts of copper were found in some of the specimens analyzed, and zinc in all except the

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[1] Bradley, H. C., *Science*, 1904, xix, 196.

[2] Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiv, 313.

[3] Dieulafait, L., *Compt. rend. Acad.*, 1880, xc, 1573.

[4] Willard, J. T., *J. Am. Chem. Soc.*, 1908, xxx, 902.

[5] Phillips, A. H., in *Papers from the Department of Marine Biology of the Carnegie Institution of Washington, Publication 251*, 1917, xi.

crayfish blood. Since the Tortugas Islands are far removed from any possible contamination of the sea water, zinc must be considered as a normal constituent of these forms. Manganese was present in all the specimens analyzed, but in varying amounts. The most remarkable occurrence, however, was that of lead in the liver of *Fasciolaria gigantea*, in quantity just sufficient to weigh, in a 20 gm. sample of dried liver, but quite enough to yield good qualitative tests.

In 1915 the presence of notable amounts of zinc in oysters was discovered almost simultaneously in two of the laboratories of the Bureau of Chemistry, U. S. Department of Agriculture. Each laboratory was working independently of the other and on quite different problems.

In the winter of 1915, Sale, in the Water Laboratory in Washington, discovered the presence and determined the quantity of zinc and manganese in samples of oysters from Chesapeake Bay. This work was done in connection with a study of the composition of the water of the Bay. At about the same time, but without knowledge of Sale's studies, the writers, working in the Denver Laboratory on the problem of the composition of oysters from polluted waters of the Atlantic coast, discovered zinc in astonishingly large amounts in each of the samples examined.

EXPERIMENTAL.

With the assistance of a number of the analysts of the Bureau of Chemistry laboratories in New York, Philadelphia, Boston, and Denver, the occurrence of zinc and also of copper in oysters from various localities on the Atlantic seaboard, the relation of zinc in oysters to the zinc content of the water in which they grew, and the ratio of zinc to copper in oysters were studied. The results of these studies are reported in the present paper. So far as is known, there have been published no other analyses of oysters or other shell fish that are used as food, with the exception of the Tortugas marine organisms already mentioned, showing the presence of zinc, arsenic, or other poisonous metals excepting copper. The analyses of Feldstein[6] in the Denver laboratory, however, show that zinc is present in considerable amounts also in many

[6] Unpublished investigations.

of the common edible marine animals such as clam, mussel, abalone, snail, shrimp, crab, and octopus.[7]

Identification of Zinc.—On account of the quantity of zinc reported by the analysts cited, it seemed important to establish the identity of the metal beyond question. To this end, an authentic sample of shucked oysters, on which a report had been made by Wichmann of 1,392 mg. per kilo, was subjected to a careful examination. Clearly positive reactions were obtained in every test. The solubility of the various salts, the color of the oxide, hot and cold, the color and solubility of the sulfide and of the ferrocyanide and phosphate were all strictly normal. Practically, the theoretical weight of anhydrous zinc sulfate was obtained from the oxide produced by the ignition of the sulfide precipitated from acetic acid solution. The same was true of the weight of pyrophosphate obtained by the conversion of the sulfate described above. The yield from 0.3792 gm. of oxide was 0.7509 gm. of anhydrous sulfate and 0.7083 gm. of pyrophosphate. The theoretical yield from zinc oxide would be 0.7521 gm. of sulfate and 0.7099 gm. of pyrophosphate.

Methods of Analysis.

No originality is claimed for the procedure followed in this study. Well known and reliable methods were adopted. The following details, developed to meet the requirements, were found to give dependable results.

1. Preparation of Sample.—At least a pint, representing the lot under examination, was thoroughly comminuted, using a form of grinder or chopper that did not cause a separation and loss of the fluid portion. Any liquid that separated was intimately mixed with the solid portion after grinding. Copper and zinc were determined in one weighed portion of the sample, using 100 gm. when practicable.

2. Destruction of Organic Matter.—This is a necessary preliminary operation for determining both zinc and copper. This was accomplished by the acid digestion process, using either a Kjel-

[7] The octopus analyzed is the canned Japanese commercial article, species not determined.

dahl flask or a silica dish, preferably the latter. Results by both methods were found to agree.

100 gm. of the well mixed sample were weighed into a silica dish, 20 cc. of 25 per cent sulfuric acid added, and evaporated on the steam bath over night. The residue was then carefully heated and the excess of sulfuric acid slowly fumed off by placing the dish in front, but not inside of a hot muffle. The organic matter was thus readily oxidized without overheating. When the excess of sulfuric acid was removed the contents of the dish were moistened with concentrated nitric acid and gradually heated. Nitric acid was added in small quantities from time to time, followed by periods of heating inside the muffle at gradually increasing temperatures until the organic matter was destroyed. Care was exercised to control the temperature of the muffle below a dull red heat at first, and to add sufficient nitric acid to prevent flaming and possible loss of zinc. The nitric acid was added most conveniently drop by drop from a pipette directly to the hot material. This method is preferred for examining oysters and similar products because a large sample can be disintegrated and reduced to ash easily and quickly without the danger of losses by frothing or by breakage of apparatus. It is not feasible to destroy the organic matter directly by burning, owing to the danger of losing both copper and zinc by volatilization.

3. Separation of Copper from Zinc.—Copper was precipitated as sulfide from a slightly acid solution with hydrogen sulfide, filtered, and the filtrate reserved for the determination of zinc.

4. Determination of Zinc.—The acid filtrate contained zinc, iron, manganese, phosphates, etc. To determine zinc, the filtrate was treated with 1 cc. of nitric acid and evaporated to dryness and the residue dissolved in 50 cc. of a reagent of the following composition: ammonium chloride, 200 gm.; ammonia, 500 cc.; water, 750 cc. The solution was heated to boiling and treated with a few cc. of bromine water, then boiled 2 or 3 minutes, filtered, and the precipitate washed with a hot solution consisting of 100 gm. of ammonium chloride and 50 cc. of ammonia to the liter. The filtrate contained all the zinc originally present in the sample. The filtrate was neutralized with hydrochloric acid and an excess of 2 cc. of concentrated acid added. The solution was then diluted to about 200 cc. and titrated hot with

a standardized solution of potassium ferrocyanide equivalent to 2 to 3 mg. of zinc per cc., using a 5 per cent solution of uranium acetate on a spot plate as an "outside" indicator. Whenever it was necessary to make a series of zinc determinations the ferrocyanide solution was standardized using a pure zinc solution of known strength containing ammonium salts and hydrochloric acid in about the same proportion as in the sample under examination. This procedure is essentially the method described by Low.[8] The details and precautions suggested in the text should be carefully observed. The results of analyses are stated in terms of mg. per kilo.

Copper may be determined in the sulfide precipitate described above by any approved method. Low's[8] iodometric method gives reliable results and for several reasons is preferred.

Results of Analysis.

The results of the determinations of zinc in oysters by the method described are given in Tables II to VI, inclusive.

Table I contains the results obtained by Sale by a different method. The presence of zinc was not anticipated by him, so that the samples were reduced to ash as a rapid means of preparing them for analysis. A portion of the zinc may have been lost in the operation by reduction and volatilization. The zinc and manganese were precipitated together as sulfides and reprecipitated with sodium carbonate and weighed as oxides. The values for zinc were obtained by difference after subtracting the figure for manganese, which was determined colorimetrically.

In all the tables the results are given for each sample analyzed for the purpose of showing the individual variations. A statement of the averages would hardly suffice to show the true status of the matter.

From the data presented it is evident that zinc is present universally in oysters, or at any rate in those grown in the Atlantic waters along the coast of the United States.

The data tabulated also show that in oysters zinc is associated invariably with copper. No samples examined in the Bureau of

[8] Low, A. H., Technical methods of ore analysis, New York, 5th edition, 1911.

TABLE I.

*Determination of Zinc in Shucked Oysters.**

Sample No.	Source.	Zinc in drained oyster flesh.
		mg. per kg.
	Shell stock from oyster house, said to be from	
8,905-H	Patuxent River, Md.	111
8,906-H	Holland Point, Md.	161
8,907-H	" " "	320
8,908-H	Rappahannock River, Va.	102
8,909-H	" " "	82
8,910-H	Fishing Bay, Md.	108
	Chester River:	
18,981-H	Bed 14, Queen Annes Co., Md.	504
18,982-H	" 39, Kent Co., Del.	247
18,984-H	" 43, " " "	394
18,985-H	" 46, " " "	459
18,989-H	" 4, Queen Annes Co., Md.	274
18,991-H	" 64, Kent Co., Del.	118
	Chesapeake Bay, lower end:	
20,520-H	Bed 15, Anne Arundel Co., Md.	46
20,521-H	" 16, " " " "	38
20,522-H	" 14, " " " "	319
	Severn River:	
20,506-H	Bed 43, Anne Arundel Co., Md.	58
20,507-H	" 23, " " " "	124
20,509-H	" 27, " " " "	620
20,515-H	" 23, " " " "	217
20,517-H	" 45, " " " "	143
20,519-H	" 45, " " " "	26
18,945-H	Rhode River, Bed 72: Anne Arundel Co., Md.	74
	West River:	
18,944-H	Bed 70, Anne Arundel Co., Md.	186
18,947-H	" 81, " " " "	281
	Patuxent River:	
18,908-H	Bed 3, St. Marys Co., Md.	70
18,913-H	" 22, Calvert Co., "	186
18,994-H	" 22, St. Marys Co., "	171
18,995-H	" 22, Calvert Co., "	169
18,997-H	" 14, St. Marys Co., "	150
19,000-H	" 31, Calvert Co., "	61
—	Pink oysters (decomposed) shipped from South Norwalk, Conn.	394
	Maximum	620
	Minimum	26

* Analyses made by J. W. Sale in the Water Laboratory, Bureau of Chemistry, 1914.

Chemistry have been found to be free from these metals. The amounts of metals present appear to have no direct relation to the body weight of the oyster nor to the quantity of ash-forming solids it contains. The presence of very large amounts of copper is usually revealed by a blue or bluish green coloration. Such oysters invariably contain large amounts of zinc, though not in uniform proportion to the copper. The blue coloration serves,

TABLE II.

*Determination of Arsenic, Copper, and Zinc in Oysters.**

Sample No.	Source.	Arsenic.	Copper.	Zinc.
		mg. per kg.	*mg. per kg.*	*mg. per kg.*
17,054-K	New York, N. Y.	0.95	86.4	556.0
17,055-K	Crisfield, Md.	None.	17.0	172.0
17,057-K	" "	0.77	17.8	133.7
17,058-K	" "	0.77	33.7	252.3
17,063-K	St. Michaels, Md.	0.84	36.4	346.5
17,064-K	New Haven, Conn.	1.24	72.9	1,115.0
17,065-K	New York, N. Y.	1.16	75.1	541.0
17,066-K	Chincoteague, Va.	1.47	11.9	171.0
17,069-K	New York, N. Y.	0.98	98.5	692.3
17,069-K	(Drained meats)	0.98	136.5	953.3
17,069-K	(Liquor)	0.77	23.9	185.8
17,070-K	New York, N. Y.	1.12	90.8	724.1
17,071-K	South Norwalk, Conn.	1.12	158.8	1,168.5
17,072-K	" " "	1.33	152.2	1,039.0
18,745-K	Boston, Mass.	Not determined.	101.2	1,090.0
20,764-L	New York, N. Y.	1.30	175.3	1,302.5

* Analyses made by H. J. Wichmann, in the Denver laboratory, January, 1915.

therefore, as a reliable indicator of the presence of large amounts of these metals. Indeed, the oysters from certain localities, such as Perth Amboy, New Jersey, and New York Harbor, have gained unfavorable notoriety because of the amounts of copper they contain, frequently excessive enough to impart to them a strong turquoise blue color and a metallic flavor, and to yield a decided copper coating to bright iron when boiled with it in dilute acid. Since the amounts of copper and zinc found in apparently nor-

TABLE III.

*Determination of Amounts of Copper and Zinc in Shucked Oysters.**

Sample No.	Source.	Copper.		Zinc.	
		Drained oysters.	Liquor.	Drained oysters.	Liquor.
		mg. per kg.	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
B-812	Jamaica Bay, N. Y.	48.8	12.8	876	60.0
B-814	" " "	92.8	11.2	540	52.0
B-816	" " "	57.6	8.0	848	16.0
B-818	" " "	80.0	11.2	616	8.0
B-820	" " "	83.2	14.4	280	Trace.
B-822	" " "	64.0	11.2	648	76.0
Maximum		92.8		876	
Minimum		48.8		280	
B-840	Great South Bay, off West Sayville, N. Y.	67.0	3.0	646	Trace.
B-842	" " " " " "	51.0	1.0	598	4.0
B-844	" " " " " "	161.0	4.0	1,102	11.0
B-846	" " " " " "	87.0	5.0	742	Trace.
B-848	" " " " " "	41.0	Lost.	534	Lost.
B-849	" " " " " "	94.0	5.0	701	10.0
B-850	" " " " " "	29.0	4.0	429	5.0
Maximum		161.0		1,102	
Minimum		29.0		429	

* Analyses made by Louis D. Elliott, in the New York laboratory, 1915.

B-851	Raritan Bay.	154.0	9.0	912	85.0
B-853	" "	128.0	4.0	768	71.0
B-852	Sandy Hook.	167.0	7.0	1,082	57.0
B-854	" "	141.0	5.0	912	69.0
A	Bridgeport, Conn., Bed 804	444.2	21.8	1,068	70.4
B	" " " 831	283.5	16.6	828	67.2
C	" " " 718	285.2	11.5	280	33.6
D	" " " 710	370.1	16.6	880	64.0
E	" " " 749	255.2	17.9	960	99.2
A	Milford, " " 674	283.5	19.2	920	81.6
B	" " " 657	261.5	11.5	1,184	67.2
C	" " " 607	371.5	15.4	1,176	81.6
Maximum		444.2		1,184	
Minimum		255.2		280	
A	New Haven, Conn.	539.0	25.0	1,363	56.0
B	" " "	257.0	8.0	1,760	37.0
C	" " "	244.0	13.0	1,357	69.0
D	" " "	270.0	16.0	1,475	84.0
E	" " "	308.0	19.0	1,411	83.0
F	" " "	180.0	13.0	870	94.0
Maximum		539.0		1,760	
Minimum		180.0		870	

TABLE III—*Continued.*

Sample No.	Source.	Copper.		Zinc.	
		Drained oysters.	Liquor.	Drained oysters.	Liquor.
		mg. per kg.	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
A	Vicinity Stony Creek, Conn., Flying Point	124.0	6.0	870	39.0
B	" " " " Grass Rock	131.0	4.0	714	24 0
C	" " " " near Cut-in-Two Island.	120.0	5.0	749	52.0
D	" " " " between Waylands and Davis Island	124.0	6.0	982	79.0
E	" " " " between Pot and Money Islands	146.0	7.0	1,014	89.0
F	" " " " between High and Crib Islands	101.0	6.0	755	40.0
G	" " " " northeast of Governor's Island	86.0	6.0	717	74.0
H	" " " " northeast of Roger's Island	94.0	5.0	595	67.0
Maximum		146.0		1,014	
Minimum		86.0		595	
A	South Norwalk, Conn.	172.0	20.0	1,315	108.0
B	" " " Town Bed	262.0	10.0	920	94.0
C	" " " Bed 1,078	290.0	10.0	1,792	80.0
D	" " " " 1,025	371.0	13.0	2,298	69.0
E	" " " " 1,118	318.0	13.0	1,789	102.0
F	" " " " 1,152	224.0	10.0	1,488	38.0
G	" " " Town Bed	179.0	12.0	1,798	107.0
I	" " " " " 42	64.0	3.0	778	62.0
J	" " " Bed 1,048	293.0	24.0	1,894	83.0
K	" " " Tallmodges Bed	173.0	5.0	1,667	82.0
L	" " " mouth of Saugatauk River	132.0	6.0	1,331	32.0
Maximum		371.0		2,298	
Minimum		64.0		778	

Maximum	371.0		2,298		
Minimum	64.0		778		

3,032-K	Chesapeake Bay, Governor's Run	30.0	Not determined.	714	Not determined.
3,033-K	" " Lumps	181.0	" "	1,162	" "
3,034-K	" " Bodkin Point, Md	214.0	" "	1,309	" "
3,035-K	" " Swan Point, Md	179.0	" "	1,197	" "
3,036-K	" " Crab Alley Creek	59.0	" "	461	" "
3,037-K	" " Hoy Bay	101.0	" "	512	" "
3,038-K	" " Holland Point, Md	76.0	" "	755	" "
3,039-K	" " Chester River, Md	111.0	" "	829	" "
3,040-K	" " West River, Swan Point, Md	111.0	" "	778	" "
3,041-K	" " Port Tobacco, Md	101.0	" "	624	" "
3,042-K	" " Fishing Bay, Md	28.0	" "	170	" "
3,043-K	" " Herring Bay, Md	68.0	" "	810	47.0
3,044-K	" " mouth of Patuxent, Md	38.0	" "	381	Not determined.
3,045-K	" " Chester River, The Cliffs	88.0	" "	522	" "
Maximum		214.0		1,309	
Minimum		28.0		170	
346-K	Potomac River, near Coles Point, Va	38.0	Not determined.	355	Not determined.
347-K	Pocomoke Sound	52.0	" "	256	" "
348-K	Potomac River, off Point Lookout, Md	48.0	Trace.	182	" "
349-K	" " near Blackistone Island, Md	54.0	"	237	40.0
351-K	Blackistone Island, Md	72.0	2.5	250	11.0
352-K	Jackson's Creek, Westmoreland Co., Va	46.0	Not determined.	307	Not determined.
3,121-K	Chesapeake Bay	20.0	" "	189	" "
3,122-K	South of Blackistone Island, Md	52.0	1.6	186	11.0
Maximum		72.0		355	
Minimum		20.0		182	

TABLE III—*Concluded.*

Sample No.	Source.	Copper.		Zinc.	
		Drained oysters.	Liquor.	Drained oysters.	Liquor.
		mg. per kg.	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
1,899-K	Near Charleston, S. C., Bulls Bay beds	35.0	Not determined.	189	Not determined.
1,900-K	" " " Edith Island beds	23.0	" "	493	" "
B-900	Perth Amboy, N. J.†	1,487.0	15.2	2,903	36.4
3,307-K	Sapelo beds, Atlantic Ocean, 80 miles south of Savannah, Ga	48.0	Not determined.	410	Not determined.
3,308-K	Near Savannah, Ga., Herb River beds	24.0	2.2	182	10.0
3,309-K	East side, Savannah, Ga., Tybee Island beds	52.0	Not determined.	317	Not determined.
3,310-K	West " " " " " "	40.0	" "	189	" "
3,311-K	Near Savannah, Ga., Thunderbolt River beds	44.0	" "	346	" "
3,312-K	" " " Daufuskie Island beds	24.0	" "	502	" "
	Maximum	52.0		502	
	Minimum	24.0		182	
3,784-K	Crittenden, Va., mouth of James River	89.0	Not determined.	643	Not determined.
3,785-K	Lynnhaven Bay, Va	25.0	1.0	253	12.0

† A sample of blue oysters. The bluest in the sample were selected for analysis. The oyster beds at Perth Amboy are off-shore from large copper refineries and other metallurgical works.

mal oysters from different localities vary so widely, and since there seems to be no uniformity in the ratio between their copper and their zinc content, it would seem that oysters are capable of taking up amounts of these metals beyond their immediate physiological needs.

There appears, moreover, to be no direct, uniform ratio between the quantity of copper and zinc in the oysters and the amount in the sea water in which they are found, although it is true, in general, that oysters contain larger proportions of the

TABLE IV.

*Determination of Copper and Zinc in Shucked Oysters.**

Sample No.	Source.	Copper in drained meats.	Zinc in drained meats.
		mg. per kg.	*mg. per kg.*
498-K	Bivalve, N. J.	30.9	779
499-K	Maurice River, N. J.	17.0	472
500-K	Parker's Bay, Stockton, Md.	8.8	146
2,802-K	Johnson's Bay, Md.	7.6	175
2,803-K	Franklin City, Va.	8.8	153
2,804-K	West Sayville, L. I., N. Y.	30.2	507
2,805-K	Maurice River, N. J.	15.1	650
2,806-K	Baldwin, L. I., N. Y.	6.3	562
2,807-K	Chincoteague, Va.	18.8	201
2,808-K	Girdle Tree, Md.	19.1	168
Maximum		30.9	779
Minimum		6.3	146

* Analyses made by Clarence L. Black, in the Philadelphia laboratory, March, 1915.

metals when grown in sea water highly contaminated with metallic wastes from smelters and other factories. The data presented in the tables show this clearly; for example, oysters dredged along the New Jersey coast near Perth Amboy and from waters farther north in New York Harbor. A notable exception to this general rule is seen in the samples collected from the vicinity of Savannah, Georgia, and analyzed in the New York laboratory (Table III, Samples 3,307-12-K). Sample 3,307-K, taken from the ocean about 80 miles south of Savannah, contained approximately twice as much copper and zinc as two other

samples (Nos. 3,308-K and 3,310-K) from beds in Savannah Harbor.

In order to secure data on the source of the zinc, the Bureau of Chemistry laboratory at New York analyzed two samples of sea water, and Feldstein, of the Bureau of Chemistry laboratory at Denver, analyzed eight samples of vegetation and organic matter that came up in the dredges with the oysters from beds

TABLE V.

*Determination of Copper and Zinc in Shucked Oysters.**

Sample No.	Source.	Copper in drained meats.†	Zinc in drained meats.‡
		mg. per kg.	*mg. per kg.*
2,741-K	Opposite Sayville, L. I., N. Y.	105	963
2,742-K	Bridgeport Gut, Conn.	362	1,050
2,745-K	Seaconet, Mass.	82	1,206
2,746-K	Wickford, R. I.	68	701
2,747-K	Waquoit, Mass.	34	872
2,748-K	Wellfleet, "	82	1,220
2,749-K	Cotuit, "	28	762
2,750-K	Bluepoint, L. I., N. Y.	26	658
2,756-K	Eastham Beds, Wellfleet, Mass.	16	964
2,757-K	India Neck, Mass.	18	984
2,761-K	Warwick Bed, Narragansett Bay, R. I.	62	400
2,762-K	Hyde Hole, " " "	94	392
2,763-K	Rumstick Bed, " " "	54	608
	Maximum	362	1,220
	Minimum	16	398

* Analyses made by C. W. Harrison, in the Boston laboratory, 1915.

† Copper determined electrolytically.

‡ Zinc weighed as oxide.

along the coast of Cape Cod (Table IV). In the sample of water taken from Great South Bay, 1 mile off-shore from West Sayville, New York, no copper or zinc in 3,500 cc. was found, though both metals were found in oysters from that region (Table III). In water from Lupatcong Creek, a tributary of Raritan Bay, Keyport, New Jersey, a trace of copper but no zinc was found in 4,000 cc. In two samples of oysters from Raritan Bay, the New York laboratory found respectively 154 and 128 mg. of copper per kilo of the drained oyster meat, and

912 and 768 mg. of zinc. The results of the analyses of the material brought up by the oyster dredges are given in Table VI. The samples were obtained personally by one of the inspectors connected with the Boston laboratory, so that their authenticity is

TABLE VI.

*Determination of Amounts of Copper and Zinc in Shucked Oysters and Marine Debris.**

Sample No.	Source.	Oysters. Meats and liquor combined.		Vegetation and debris accompanying the oysters in the dredge.†	
		Copper.	Zinc.	Copper.	Zinc.
		mg. per kg.	*mg. per kg.*	*mg. per kg.*	*mg. per kg.*
781	Wellfleet, Mass.....	14	705	Slight trace.	89
782	" "	10	772	" "	84
783	" "	16	224	No sample.	No sample.
784	" "	131	1,026	" "	" "
785	" "	26	946	Slight trace.	50
786	Cotuit, Mass.	34	877	" "	108
787	" "	8	477	None.	47
788	" "	Not analyzed.		Slight trace.	54
789	Osterville, Mass.....	18	529	Trace.	56
790	" "	24	614	"	106
Maximum		131	1,026		108
Minimum		8	224		47

* Analyses made by L. Feldstein, in the Denver laboratory, June, 1917.

† The water from which these oysters were dredged is probably as free from pollution as any where oysters are produced commercially. Wellfleet is on the west coast of Cape Cod, and Cotuit and Osterville are on the south coast of Barnstable County, all remote from any large city or metallurgical works.

definitely established. The amount of metal in the organic matter itself is doubtless considerably larger than that indicated by the figures recorded because a quantity of sand, bits of shell, and other debris with which it was associated were weighed in with each sample analyzed.

Biological Significance of Zinc in Oysters.

In the course of this study, the writers have made no attempt to determine the physiological or biological significance of the zinc found in oysters nor to correlate the chemical data with the physiology of the mollusks. However, the finding by Feldstein of zinc in the organic matter which was dredged up with oysters may be regarded as supporting the suggestion made by Mendel and Bradley that this metal reaches the mollusk as a constituent of some one or other of the organisms upon which the mollusks feed. If this hypothesis can be substantiated further, it may furnish the key to the explanation of the lack of correlation observed in this investigation between the zinc and copper content of the oysters, and the zinc and copper content of the sea water in which the oysters grew. It may be that the oysters are rich in zinc when their food contains a considerable number of organisms, whatever they may be, that are rich in zinc, irrespective of the zinc content of the sea water, and one would then expect that the zinc content of the oyster is an indication of the character of its food rather than of the character of the sea water in which it grows. Mendel and Bradley[9] showed that the zinc present in the blood and liver of *Sycotypus* is undoubtedly combined with protein much as copper is combined in hemocyanin; and they have suggested the name "hemosycotypin" for the compound. In oysters it is probable that the zinc also occurs in the blood as well as in the body substance since the New York and Denver laboratories invariably found zinc, as well as copper, in the liquor drained from the oysters examined. The quantity of blood of the animal comprised in the liquor is variable and not readily determined, but it is probably always present to some extent in the drained liquid as a result of injuries to the oysters when shucked. Whether or not the zinc in oysters is combined with protein as in "hemosycotypin" was not investigated in the present study. Since, however, it has been shown that zinc is found in marine animals more commonly than supposed by Mendel and Bradley, it might be well to substitute some more general term, such as hemocuprazin or hemozincin, for the term hemosycotypin suggested by them.

[9] Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1906–07, xvii, 167.

SUMMARY.

Zinc is present universally in oysters, at least in those grown in Atlantic waters.

There is no direct relation between the zinc content and the body weight of the oysters, nor uniformity of ratio of zinc to copper, nor correlation between the zinc content of the oysters and the water in which they grew.

The vegetation and organic matter dredged up with oysters in the single locality studied contained notable quantities of zinc and in some instances traces of copper.

As in *Sycotypus*, zinc is probably always associated with copper.

It seems probable that zinc, as well as copper, can be absorbed and retained in the tissues of the oysters in quantities far in excess of functional requirements, especially in oysters grown in waters badly polluted with metallurgical and factory wastes.

The writers gratefully acknowledge the assistance rendered by Carl L. Alsberg, Chief of the Bureau of Chemistry, and by Clarence L. Black, Leonard Feldstein, Louis D. Elliott, and C. W. Harrison, analysts, respectively, in the Philadelphia, Denver, New York, and Boston laboratories of the Bureau of Chemistry, also by the Chemist in Charge and the Inspector of the Boston laboratory in securing authentic samples.

THE NUTRITIVE VALUE OF YEAST PROTEIN.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

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The idea of the use of yeast as a source of food protein for man and the higher animals is not a new one; it has, however, been given renewed emphasis by the exigencies of the food situation during the war. Yeast is a highly nitrogenous by-product of the fermentation industries which heretofore has been largely wasted. It also presents the possibility of a synthetic production of protein from exceptionally simple compounds independent of seasons and crops.

More recently the remarkable efficacy of the ordinary yeasts used in the manufacture of alcoholic beverages and also in bakeries in supplying water-soluble vitamine has been demonstrated.[1] This property must be regarded as something apart from the nutrient value of the yeast protein as such.

In the case of both adult men and animals the utilization of the nitrogenous components of yeast as judged by the "coefficient of digestibility" and the nitrogen balance has repeatedly been reported as good, although the statements on this point are not

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] The earlier literature of the subject is reviewed in our paper on The Rôle of Vitamines in the Diet, *J. Biol. Chem.*, 1917, xxxi, 149. See also Hawk, P. B., Fishback, H. R., and Bergeim, O., *Am. J. Physiol.*, 1919, xlviii, 211. For the use of yeast in the diet of growing mice see Thompson, H. B., and Mendel, L. B., *ibid.*, 1918, xlv, 431.

uniformly unanimous.[2] For example, Funk, Lyle, and McCaskey[3] assert: "A large part of the yeast nitrogen apparently has no food value. It is badly assimilated."

The nutritive needs of a growing animal make the most exacting demands for physiologically adequate protein; hence feeding experiments during growth are particularly advantageous for determining the dietary usefulness of nitrogenous nutrients. We are not aware of any records of prolonged growth when yeast was used as the sole source of protein. Funk, Lyle, and McCaskey[3] state that "young rats can live on yeast as the sole nitrogen source for quite a long while, although it has not yet been proven whether they can subsist on it indefinitely."

The actual protocols recorded by Funk[4] are neither convincing nor conclusive. One of the two experimental rats receiving its nitrogen in the form of yeast died with edema after 44 days. The other appears to have grown normally for 52 days. Funk states (p. 9) without submitting further evidence: "Yeast cannot be regarded as a good substitute for protein. There is even a slight indication of a toxic action."

We have kept rats successfully for more than a year covering the period of growth, upon a diet in which yeast furnished the sole source of nitrogen as well as water-soluble vitamine. The food mixtures consisted of:

	Per cent	*Per cent*
Dried yeast	30*	40†
Salt mixture‡	4	4
Starch	43	33
Butter fat	9	9
Lard	14	14

* Yeast obtained from the Schlitz Brewing Co., Milwaukee, Wis.

† Yeast obtained from the Hinckel Brewery Co., Albany, N. Y.

‡ For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

The growth curves are shown in the chart.

[2] For a bibliography of the subject see Hawk, P. B., Smith, C. A., and Holder, R. C., *Am. J. Physiol.*, 1919, xlviii, 199. Funk, C., Lyle, W. G., and McCaskey, D., *J. Biol. Chem.*, 1916, xxvii, 173. Schill, E., *Biochem. Z.*, 1918, lxxxvii, 163.

[3] Funk, C., Lyle, W. G., and McCaskey, D., *J. Biol. Chem.*, 1916, xxvii, 173

[4] Funk, C., *J. Biol. Chem.*, 1916, xxvii, 1 (see p. 9, Experiment 6).

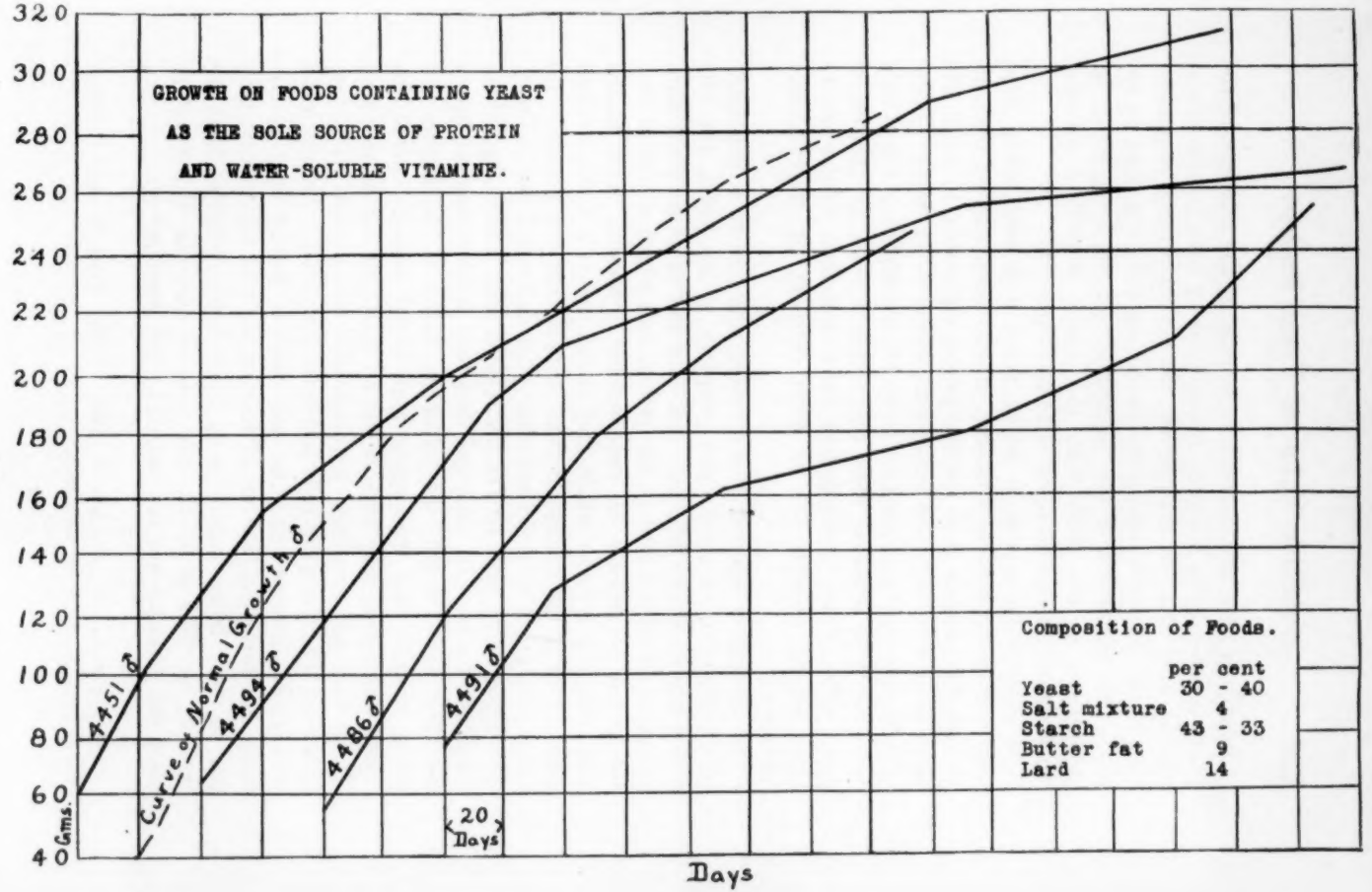
GROWTH ON FOODS CONTAINING YEAST
AS THE SOLE SOURCE OF PROTEIN
AND WATER-SOLUBLE VITAMINE.
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
Gms.
4451 ♂
Curve of Normal Growth ♂
4494 ♂
4486 ♂
4491 ♂
20 Days
Days
Composition of Foods.
per cent
Yeast 30 - 40
Salt mixture 4
Starch 43 - 33
Butter fat 9
Lard 14

CHART I.

Inasmuch as a not inconsiderable part of the nitrogen of the yeast is non-protein nitrogen, represented by such compounds as nucleic acid and its derivatives, the actual protein concentration of the successful diet must have been even less than 18 per cent. An estimation of the digestibility of the food, tested by comparison of the feces with the intake showed a nitrogen "utilization" in several cases of 74 to 83 per cent.

In other experiments in which yeast was used solely as a source of water-soluble vitamine, it had been noticed that although the animals thus fed had grown vigorously to adult size they were, with very few exceptions, found to be sterile. Some of these rats were examined for us by Dr. Donaldson of the Wistar Institute, who reported that the testes showed an absence of germ cells. Other investigators have described similar conditions among animals fed on diets deficient in water-soluble vitamine.[5]

To determine whether this sterility was due to a deficiency in vitamine, or to some other property of the yeast, the rats which had grown to full adult size on a diet in which yeast composed 30 to 40 per cent of the ration, and which therefore contained an abundance of water-soluble vitamine, were mated with vigorous females which had grown up on a normal mixed diet. Two of the animals proved to be fertile but produced inferior young; while two of the other yeast-fed rats failed to breed. The latter were sent to Dr. Donaldson who kindly had them examined for us. He reported that they were somewhat heavy for their body length, the refractive index of the serum was below normal, and the organs were about normal, except the testes which were only one-quarter to one-third the normal size. These were not examined histologically, but a gross inspection indicated that the germ cells had been destroyed. Although the number of observations is far too small to justify final conclusions, the fact that two out of four of the rats which were long fed on very large quantities of yeast bred, indicates that infertility on diets in which only a small quantity of yeast supplied the vitamine was not caused by the yeast *per se*.

[5] Drummond, J. C., *Biochem. J.*, 1918, xii, 25. Funk, C., and Douglas, M., *J. Physiol.*, 1913–14, xlvii, 475.

The fact that animals tolerate such large quantities of a food product so unique as is yeast in its chemical make-up seems to exclude the presence of a toxic factor. Consequently there is little occasion to fear any detriment from such doses of yeast as are employed for therapeutic purposes.

STUDIES ON ENZYME ACTION.

XVII. THE OXIDASE, PEROXIDASE, CATALASE, AND AMYLASE OF FRESH AND DEHYDRATED VEGETABLES.

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(Received for publication, April 16, 1919.)

Outline of Investigation.

The question of food hormones has become prominent in recent years in connection with the development of certain pathological conditions due to the lack of the chemically unknown constituents in diets apparently adequate in protein, fat, carbohydrate, mineral components, and calories. Under the term food hormones may be included vitamines, antiscorbutic property, growth-producing property, etc. Considerable evidence is being accumulated in regard to the treatment of foodstuffs which results in the loss of one or more of these food hormones. Recently published work on the loss of the antiscorbutic property for guinea pigs of certain vegetables on dehydration indicates that simple chemical or physical treatment may cause the destruction of these vitally important factors.[1] Until positive evidence to the contrary is forthcoming, the changes in the food hormones which take place on their "inactivation" may be considered to be chemical in character. The same standpoint will be taken with regard to their chemical nature as

[1] Holst, A., and Frölich, T., *Z. Hyg. u. Infectionskrankh.*, 1912, lxxii, 1; 1913, lxxv, 334. Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1917, x, 141. Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1918, xxxv, 487. Givens, M. H., and Cohn, B., *ibid.*, 1918, xxxvi, 127. Delf, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 448. Givens, M. H., and McClugage, H. B., *J. Biol. Chem.*, 1919, xxxvii, 253.

was taken in the chemical study of enzymes.[2] Food hormones are not considered necessarily to be chemical individuals. It is sufficient to ascribe more or less definite food hormone properties to certain groupings in the molecules, possibly protein, and the loss of such properties to a change (possibly tautomerism or rearrangement) in such groupings.

The changes in foodstuffs which result in different nutritive values do not appear in the ordinary chemical analyses. In order to obtain if possible some evidence of such changes, and also to follow any chemical changes taking place in such simple treatments as dehydration, this series of studies was undertaken. It includes a general enzyme study, a study of certain vegetable proteins, such as the determination of their isoelectric points, etc. (Cohn and coworkers), and an attempt to follow carbohydrate changes on dehydration. This paper will take up the results of the enzyme studies.

Changes in enzyme action (activation as well as inactivation) may be brought about by comparatively simple treatments. Changes in nutritive values of foodstuffs may also result from simple treatments. It is not assumed that the two sets of phenomena due to enzymes and to food hormones are due to the same chemical grouping or even molecule. It was considered that a general study of the enzymes of a number of vegetables would give results which, when compared with the physiological actions of the same vegetables would show similarities and differences which might aid in throwing light on the chemical nature of the substances involved in the various actions.

The enzymes oxidase, peroxidase, catalase, and amylase were studied in potatoes, tomatoes, carrots, white and yellow turnips, and cabbage. Fresh, air-dehydrated, and vacuum-dehydrated vegetables were used.

HISTORICAL.

Oxidases have been found to occur in many vegetables. There is no one method or reagent for testing for their presence. A number of different reagents have been used, such as various phenols, amines, etc. Peroxidases are almost universally dis-

[2] Falk, K. G., *Proc. Nat. Acad. Sc.*, 1916, ii, 557; *J. Biol. Chem.*, 1917, xxxi, 97; *Science*, 1918, xlvii, 423.

tributed in plants. Just as with oxidases, there is no general test for them. Catalase also is widely distributed in a number of different plants.

Quantitative measurements of the amounts of these enzymes, especially catalase, have been made at different times. The effect of the hydrogen ion concentration on their actions does not seem, however, to have been studied to any extent. Attempts at purifying the enzymes have also been described.

Although amylase has not been studied in many vegetables or plants, the work of Sherman and his associates[3] on malt, pancreatic, and *Aspergillus oryzæ* amylases has thrown much light on the conditions for their actions and the properties of highly purified materials. A careful study on potato amylase was published recently by Doby.[4]

Preparation of Materials.

The fresh vegetables were scraped (carrots and turnips), peeled (potatoes), or in the case of cabbage the outer leaves removed. They were then weighed, ground finely in a meat chopper, and squeezed through muslin by hand, until as much extract as possible was obtained. The tomatoes were cut and then squeezed similarly. The extract was strained through fresh muslin a second time to remove the coarser particles which had been forced through the first muslin. The residue was weighed and its moisture content determined by drying to constant weight at 100°. The volume of the extract was measured and its hydrogen ion concentration determined.

The air-dehydrated vegetables were prepared by blowing a current of heated air (40–65°) over the vegetable cut in thin slices for 18 to 60 hours until the substances were sufficiently dry. Some preparations (California) were bought on the open market. The vacuum-dehydrated products were prepared by heating the sliced vegetables at 55–65° at 40 to 50 mm. pressure for 8 to 12 hours. Moisture was determined in these products by drying to constant weight at 100°, nitrogen by the Kjeldahl method, and ash by ignition to constant weight.

[3] Sherman, H. C., and associates; numerous papers in *J. Am. Chem. Soc.*, 1910–1919.

[4] Doby, G., *Biochem. Z.*, 1914, lxvii, 166.

After considerable experimentation, the following method of testing the dehydrated vegetables was adopted. To 14 gm. of dehydrated vegetable were added 180 cc. of water (the average amount lost on dehydration) at 20–25°. The mixture was allowed to stand for 20 minutes and then passed through the food chopper. The chopped vegetable was then returned to the water in which it had been soaked, ground with it in a glass mortar and allowed to extract for 30 minutes, squeezed through muslin, and treated as with fresh vegetables. This part of the investigation dealt mainly with enzymes. In attempting to prepare a juice from the dehydrated vegetables comparable to fresh juice, it was found that no more enzyme was extracted by treatment in the manner described for 2 hours or even longer than after 30 minutes treatment. This juice after longer extraction when tested with the solid residue re-added did not give as strong enzyme tests as the dehydrated vegetable ground in a mortar for a shorter period of time with water and tested immediately. Apparently the enzymes were not extracted as readily from the dehydrated products as from the fresh, and if left in contact with the water for longer periods of time deteriorated or lost their activity to some extent. Differences were found as well in the enzyme properties of products dehydrated by different processes, as will be pointed out later.

The properties of the dehydrated vegetables and of the juices prepared from fresh and dehydrated products may be summarized briefly. The appearance of the vacuum-dehydrated product was in every case superior to that of the air-dehydrated; better color, texture, etc. The chemical analyses did not show any definite differences. The moisture contents varied between 7 per cent and 20 per cent (highest with tomato preparations) for the various substances. No regularity was apparent except that longer dehydration yielded more anhydrous products. The ash percentages varied similarly in a more or less irregular manner. As these variations were apparently accidental, the results will not be given in detail.

The hydrogen ion concentrations of the juices were determined colorimetrically, using the Clark and Lubs indicators[5] chiefly and also their solutions as standards. The indicators used included dibromocresolsulfonephthalein, dibromothymolsulfonephthalein, phenolsulfonephthalein, o-cresolsulfonephthalein, phenolphthalein, thymolsulfonephthalein, thymolphthalein, methyl orange, cochineal, methyl red, and rosolic acid.

[5] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191.

Methods of Testing for Enzymes.

The following reagents were used.

For oxidase.—Röhmann-Spitzer reagent.[6] 1 per cent aqueous p-phenylenediamine hydrochloride and 1 per cent α-naphthol in 50 per cent ethyl alcohol, kept separately and equal volumes mixed just before using. 5 drops of reagent were added to 2 cc. of juice or mixture and the color developed in ½ minute and in 15 minutes was noted. A very faint pink color was marked 1 (+ or −) in the tables, a deeper shade 2, and a deep red-purple 3. This reagent is more delicate than guaiaconic acid or α-naphthol and was therefore adopted for oxidase. Its color could also be more readily detected through the natural color of the juice.

For peroxidase.—1 per cent α-naphthol in 50 per cent ethyl alcohol. 5 drops of this solution and 2 cc. of 3 per cent hydrogen peroxide solution at the same hydrogen ion concentration as the mixture tested were added to 2 cc. of the juice or mixture, and the color developed in ½ minute and in 30 minutes was noted. The first pink or lavender was 1 (+ or −), a deeper lavender 2, a deep blue-purple 3. Blank solutions gave no test with the reagent in 1 hour at pH 2 to 11. The Röhmann-Spitzer reagent was found to be too delicate for peroxidase, differences at different hydrogen ion concentrations and with fresh and dehydrated products not being readily distinguishable.

For catalase.—Evolution of gas and production of foam on addition of hydrogen peroxide in peroxidase test were noted and indicated as before.

It was found that with dehydrated vegetables, 0.5 gm. ground in a mortar with 7 cc. of distilled water, transferred to a test-tube and tested for these enzymes, frequently showed greater actions than a mixture of the juice prepared as described from dehydrated vegetables plus the solid residue from the juice.

For amylase.—The Lintner method,[7] modified slightly for the purposes in view, was used. An experiment consisted of a series of test-tubes each containing 0.5 cc. of the vegetable juice or mixture and 2 cc. of freshly prepared (Lintner) starch solution.

[6] Röhmann, F., and Spitzer, W., *Ber. chem. Ges.*, 1895, xxviii, pt. 1, 567.

[7] Lintner. C. J., *J. prakt. Chem.*, 1886, xxxiv, 378.

Immediately after making up the mixtures (for the blanks) or after 2 hours incubation at 37° (or other time interval) varying amounts of Fehling solution differing by 0.5 cc. were added to the test-tubes, these immersed in boiling water for 10 minutes, and then compared. The extreme amounts of Fehling solution used were 2 cc. and 11 cc. The end-point, or amount of action, was taken to be the last tube which failed to show any blue color. In a series of test-tubes ranging from those in which the yellow precipitate was so finely divided that it did not settle, through no blue color to a deep blue, the end-point could readily be obtained. Duplicates were run throughout.

In order to obtain the actions of a vegetable juice at different hydrogen ion concentrations, the following experiments were performed. (1) A preliminary series without incubation with 3 to 11 cc. of Fehling solution for the fresh juice and 0.50 to 5.00 cc. for the dehydrated vegetable juice in order to find the approximate end-points. (2) Blank and incubation experiments at hydrogen ion concentrations ranging from pH 2 to 11. The juice and the starch solution were brought to the requisite acidities and mixed. Sets of twelve tubes were used in each series of hydrogen ion concentrations and the amounts of Fehling solution varied progressively. (3) A series with the boiled juice at its natural hydrogen concentration. (4) A series with 0.25 cc. of juice plus 0.25 gm. of residue in place of the 0.50 cc. of juice at its natural hydrogen ion concentration.

The results were recorded in terms of cc. of Fehling solution reduced. No attempt was made to calculate the actions according to the Lintner scale. The actual amount of material in the juice was not known and varied considerably.

The Wohlgemuth[8] method for amylase was found to be unsatisfactory for this work for the following reasons: (1) The difficulty of determining the end-point in some of the vegetable juices, such as the carrot and yellow turnip, which are colored. Instead of obtaining a series of clear colors from orange or yellow through purple to blue, greenish, greyish, and dirty brown mixtures were obtained. (2) The vegetable extracts themselves contained varying amounts of starch so that hydrolysis of the added starch

[8] Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 1.

could not be taken as an indication of the activity unless correction was made for the starch present in the extracts. This correction was obviated in the Lintner method in which amylase action was measured by the total reducing products whether produced by the action of the amylase on the added starch or that naturally present in the extract. (3) In some of the vegetables, difficulty was caused by the presence of some chemically unsaturated or iodine-combining compound which united with the iodine and prevented the development of the blue color with the starch. It was found that 1 cc. of carrot juice combined with 2 drops of 0.1 N iodine solution in 5 minutes, 1 cc. of yellow turnip juice with 6 drops, 1 cc. of potato juice with 3 drops immediately, with 6 drops in 5 minutes, and with considerably more on longer standing, 1 cc. of cabbage juice with 4 drops immediately and more on standing.

Experimental Results.

	Percentage of total wt. as juice.	Specific gravity of juice.
Cabbage (fresh)	45–60	1.028–1.031
" (vacuum-dehydrated)		1.025
" (air-dehydrated)		1.018
Carrots	25–30	1.040–1.044
" (vacuum-dehyrated)		1.020
" (air-dehydrated)		1.025
Yellow turnips	45	1.040–1.043
White " (fresh)	45–55	1.025–1.035
" " (vacuum-dehydrated)		1.025
" " (air-dehydrated)		1.025

Only with carrots was a marked difference apparent in the specific gravities of the juice from the fresh and the dehydrated products.

The hydrogen ion concentrations of all the vegetable juices, fresh as well as dehydrated, prepared as described, were found to be in the neighborhood of pH 6, with the exception of tomato juice which was very close to pH 4.

There was, however, a marked difference observed between the juices of fresh and of dehydrated products. In order to bring the hydrogen ion concentrations of the juices to various values

(between pH 2 and 12) normal acid and alkali were added. The amounts so added to a definite volume of juice to give definite hydrogen ion concentrations when plotted against these values (in terms of pH) give the titration curves of the substances whose forms probably depend upon the properties of the protein substances present. It was found in a comparison of the fresh and dehydrated vegetable juices that a change had occurred in the sense that less acid and more alkali were required to bring the juices of the dehydrated products to the same hydrogen ion concentrations than with the fresh. There was a greater change with the air-dehydrated than with vacuum-dehydrated products. For instance, with 100 cc. portions of the juices obtained from fresh, vacuum-dehydrated, and air-dehydrated white turnips, to bring them to pH 3 required 4.86 cc., 3.33 cc., and 2.17 cc. N HCl solution respectively, and to bring them to pH 9 required 1.43 cc., 2.67 cc., and 4.67 cc. N NaOH solution respectively. These changes were observed for a number of vegetables, but the results are not considered to be quantitative enough to warrant publication in detail. The qualitative observation must be emphasized, however, that there are definite changes brought about in dehydration as shown by the titration curve analyses, probably in the character of the protein material. In the paper by Cohn and his coworkers are given more quantitative data for potato and tomato juices and preparations showing conclusively that even very mild treatments influence the properties of the substances.

The results of the enzyme tests are given in Tables I to IV.

The enzyme reactions of all the vegetable juices studied were destroyed by heating to boiling for several minutes.

The oxidase reactions with carrot, yellow and white turnip, and potato juices were greatly increased on dilution. Evidently some substance (chemically unsaturated) was present which combined with the oxygen preventing it from acting on the reagent. The peroxidase reaction did not show this increased action on dilution. If oxidase and peroxidase are due to the same, or at least similar, grouping, then the addition of the hydrogen peroxide would account for the difference in behavior on dilution. The oxidase and peroxidase results are not quantitatively comparable since a much more sensitive reagent was used with the former.

There is no well defined hydrogen ion concentration for maximum action with oxidase, peroxidase, and catalase. In general terms, the actions were better in the more alkaline solutions, best on the average between pH 7 and 10. They were inhibited in acid solution, pH 2 and 3 for oxidase and peroxidase, and pH 4 for catalase, except in the case of the tomato. Inhibition occurred with these enzymes in some cases at pH 11 or 12.

With regard to the changes brought about by dehydration on the behavior of these three enzymes, the vacuum-dehydrated cabbage and carrot gave stronger oxidase reactions than did the

TABLE I.

Oxidase Tests on Vegetable Juices.

pH	Cabbage.								Carrot.				Yellow Turnip.			
	Fresh.		Fresh. 1:16		Vacuum-dehydrated.		Vacuum-dehydrated. 1:16		Fresh.		Fresh. 1:16		Fresh.		Fresh. 1:16	
	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.
2	0	0			0	0	0	0			0	0	0	0	0	0
3	1−	1	1*r*	1	1*r*	1	2−	2	0	0	1	1			1−	1−
4	1+	3*r*	1*r*	1	1−	1+	2−	2	1*r*	0	1	1+			1	1+
5	1+	3*r*			1	2	2−	2	2*r*	3*r*	1	1+	0	0	1	1+
6	1+	1+	1+	1+	1	2	2−	2	1	3*r*	2	2			1	1+
7	1+	2			1	2	1−	2	1	3*r*	2	2			1	1+
8	2−	2			1	2	2−	2+	1	3*r*	2	2	0	0	1	1
9	0	0	0	0	1+	2	2−	2+	1	3*r*	2	2			1	1
10					1	2	1	2	1	2	2	2			1+	1+
11					0	0	1	2	0	1+	2	2	0	0	2	2

	¼ min.	15 min.		¼ min.
Juice (air-dehydrated) pH 6	0	0	Juice (vacuum)	0
Solid residue (fresh)	1	3	" (air)	0
Solid residue (vacuum)	1	3	Solid (fresh)	2
Solid residue (air)	1	2	" (vacuum)	1
			" (air)	2

* *r* indicates that the reaction was given on the surface where the reagent first came in contact with the liquid but disappeared on shaking or mixing the solution.

TABLE I—*Concluded.*

pH	White Turnip.								Potato.						Tomato.	
	Fresh.		Fresh. 1:16		Vacuum-dehydrated.		Vacuum-dehydrated. 1:4		Fresh.		Fresh. 1:8		Vacuum-dehydrated.		Fresh.	
	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.	¼ min.	15 min.
2	0	0														
3					0	0	1−	1−	0	0	0	0			0	0
4			1	2	1−	1+	1+	2−	1	1	1	2			1	1
5	0	0	2	2	1	2	1+	2	1	1	1	2+	0	0		
6			1+	2	1	2	2	2	1	2	1+	2+	0	1		
7			1	2	1	2	2	2	1	2	1+	2+	0	1		
8	0	0	1+	2	1	2	2	2			1+	2+				
9			1	2	1+	2+	2	2+			1+	2+				
10					1	2	2	2+			1+	2+				
11	0	0	0	0	0−	1−	1	1+			2	2+				

	¼ min.	15 min.		¼ min.	15 min.	
Juice (air) pH 6	0	0	Juice (air 40).....	0	0	Juice (vacuum) 0
Solid (vacuum)	1−	1−	" (air 60).....	1	1	
" (air)	0	2	Solid (fresh).....	1	1+	Juice (air) 0
			" (vacuum)..	0	2+	

fresh. In every other case the enzyme action was less in the juice from the dehydrated vegetable than in that from the fresh. With regard to the materials prepared by the two methods of dehydration, vacuum and air blast, the latter gave substances in which the enzymes were destroyed more completely than the former. It is possible that part of this inhibition or destruction of enzymic property was due to changes in the material resulting in differences of extraction. However, against this view is the fact that enzyme tests on the solid residues from the extractions also showed that air dehydration caused greater destruction of the enzymes than did vacuum dehydration.

Oxidase could not be studied in tomato and potato juices at hydrogen ion concentrations less than those indicated because of the interference by the color of the juices.

TABLE II.

Peroxidase Tests on Vegetable Juices.

pH	Cabbage.								Carrot.				Yellow Turnip.			
	Fresh.		Fresh. 1:16		Vacuum-dehydrated.		Vacuum-dehydrated. 1:16		Fresh.		Fresh. 1:16		Fresh.		Fresh. 1:16	
	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.
2	0	0			0	0										
3	2	3	1−	1+	1+	2−	0	0					0	0	0	0
4	2	3	1−	1	1+	2−	0	1−	1	2	0	0	1	2	1−	2−
5	2+	3			1+	2	0	1−	1	2			2+	3+	1−	2
6	2+	3	1−	1+	1+	2	0	0	1+	2+			2+	3+	1−	3
7	2+	3			2−	2+			1+	3	0	0	2+	3+	2−	3+
8	3	3+			2	3			1+	3			2+	3+	2−	3+
9	3	3+	1−	2+	3−	3			1+	3			2+	3+	2	3+
10	2	3+			2+	3			1+	3	0	0	2+	3+	2+	3+
11	2	3+	1−	2+	0	2−			1	2			3	3+	2+	3

Juice (air), pH 2–11 0

Juice (air).... 0
Solid (fresh).. 2+
" (vacuum) 2
Solid (air).... 0

pH	White Turnip.								Potato.				Tomato.	
	Fresh.		Fresh. 1:16		Vacuum-dehydrated.		Vacuum-dehydrated. 1:16		Fresh.		Fresh. 1:8		Fresh.	
	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.
2							0	0	0	0			0	0
3	0	0			0	0	0	1−	0	1			1	2
4	2	3	0	1	1+	2	0	1−	1	1+	0	0	1	2
5	2+	3+			1+	2	0	1	2	2+	1−	1	1+	2+
6	2+	3+	0	2	1+	3	0	1+	2	3+	1−	1+	2	3
7	2+	3+	0	2+	2−	3	0	1+	3	3+	1−	1+	2	3
8	2+	3+			2	3	0	1+	3	3	2	2	2	3
9	2+	3+	0	3−	3	3	1	2			2	2	1+	2+
10	2+	3+	0	3−	1	2+	1	1+			2−	2	1+	2+
11	1	3	0	0	0	0	0	0			2−	2	1	1+

Juice (air)..... 0 (pH 6)
Solid (fresh)..... 3
" (vacuum) . 3
" (air)..... 3

Juice (vacuum)..... 0
Juice (air)... 0
Solid (vacuum)..... 2+
Solid (air).. 0

Juice (vacuum).... 0 (pH4)
Juice (air) 0 (pH4)

The amylase results as given are corrected for blanks. The average blank values are also given in the table. These values were practically the same whether determined initially without the addition of starch, or after boiling the enzyme solution, adding starch and testing immediately or after 2 hours incubation. The results are given in terms of number of cc. of Fehling solution reduced by ½ cc. of vegetable juice action on 2 cc. of the starch solution in 2 hours at 37°. 1 cc. of Fehling solution was equivalent to approximately 10 mg. of cuprous oxide on complete reduction. On the average, 2 cc. of the starch solution gave 0.06 mg. of cuprous oxide on testing. This amount was negligible in considering the results.

Well defined maxima in the amylase actions were observed with cabbage, carrot, and white turnip juices at about pH 6. With yellow turnip juice, the optimum action extended from pH 4 to 7.

TABLE III.

Catalase Tests on Vegetable Juices.

pH	Cabbage.				Carrot.		Yellow Turnip.				Potato.					
	Fresh.		Vacuum-dehydrated.		Fresh.		Fresh.		Fresh. 1:16		Fresh.		Fresh. 1:2		Vacuum-dehydrated.	
	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.
2																
3																
4	0	0			0	0	0	0	0	0	0	0	0	0		
5	1−	1−			1	1	0	1	1	1	1	2	0	1	0	1−
6	3	3			1+	2	2+	3+	1	1+	3	3	1	2	0	1−
7	3+	3+	0	0	2	3	2+	3+	1	1+	3	3	2	3	1	1+
8	3+	3+	1	1+	2	3	2+	3+	1	1+	3	3	1	2+	1	1
9	3+	3+	1	1+	2	3	2+	3+	1	1	3	3	1	1+	1	1
10	3+	3+	1	1+	2	3	2+	3+	1	1	2	2	1−	1+	1	1
11	1	1	1	1+	2	3	2	2+	0	0	0	0	0	0	1	1
6	Juice (air)		0	1	Juice (vacuum and air).....0 Solid (vacuum).....2+ Solid (air)....1−						Juice (air) pH 6 ..... 1 Solid (vacuum)..... 1 Solid (air)..... 1					

TABLE III—*Concluded.*

pH	White Turnip.								Tomato.		
	Fresh.		Fresh. 1:16		Vacuum-dehydrated.		Vacuum-dehydrated. 1:4		Fresh.		
	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	½ min.	30 min.	
2									1+	2	
3									1	2	
4	0	0	0	0					2	2+	
5	0	1			0	0	0	0	2	2+	
6	1+	2	1−	1−	1	1	0	1−	3	3	
7	2+	3+	1−	1−	1	1	0	1−	3	3	
8	2+	3+			1	1	1−	1−	3	3	
9	2+	3+	1−	1−	1	1	1−	1−	3	3	
10	2+	3	1	1	1	1	1−	1−	3	3	
11	1+	2	1−	1−	1−	1	1−	1−	2−	2−	

		½ min.	30 min.			
	Juice (air)...	0	0		Juice (vacuum).....	0
	Solid residue (vacuum).....	1	2+		" (air).....	0
	Solid residue (air)...	1	1−		Solid (fresh).....	1
					" (vacuum).....	1
					" (air).....	1

These optimum actions are of interest in comparison with the optimum actions described by Sherman, Thomas, and Baldwin,[9] pH 7 for pancreatic amylase, pH 4.4 to 4.5 for malt amylase, and pH 4.8 for *Aspergillus oryzæ* amylase. The optimum hydrogen ion concentrations for the vegetable juice amylases coincides with the natural hydrogen ion concentrations of these juices. Testing mixtures of the extract and the solid residue of the cabbage and yellow and white turnip gave results which indicated that two-thirds or more of the total activity was contained in the juice as prepared.

A comparison of the values of the blanks and the maximum actions shows that the larger the blank the greater the action.

[9] Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.

In other words, the greater the amount of amylase in the juice, the greater the amount of reducing substance in the vegetable, or the greater the action of the amylase in the vegetable itself. Potato juice, when tested for amylase by the method used with the other vegetable juices, gave an end-point which was so uncertain and difficult to determine that a different method had to be employed for it. These results will be communicated in a later paper.

TABLE IV.

Amylase Tests on Vegetable Juices.

	pH	Cabbage.			Carrot.		Yellow Turnip.	White Turnip.	
		Fresh.	Vacuum-dehydrated.	Air-dehydrated.	Fresh.	Vacuum-dehydrated.	Fresh.	Fresh.	Vacuum-dehydrated.
	3	0	0		0		0.20	0.30	0.15
	4	1.65	0.50		0.25		2.70	1.30	0.65
	5	2.15	1.00		0.25		2.45	1.55	0.65
	6	2.40	1.25	0.80	0.60	0.15	2.30	2.40	0.65
	7	1.90			0.25		2.70	1.30	0.15
	8	0.90	0		0.25		0.70	0.80	
	9	0.40			0		0	0.20	
	10	0						0	
Blanks (mean)		4.60	3.00	1.20	2.50	1.60	7.30	3.70	3.35
Maximum actions.....		2.40	1.25	0.80	0.60	0.15	2.70	2.40	0.65

The effect of dehydration on the amylase content is the same as with the other enzymes, decreased action in every case, but greater decreased actions with air dehydration than with vacuum dehydration.

A number of preliminary experiments were carried out with the vegetable juices in the presence of different salts in which the Wohlgemuth method was used. Little difference was found in the amylase actions with the added salts, so that it may be assumed that the concentrations of the electrolytes in the natural juices were sufficient to give very nearly optimum conditions.

CONCLUSIONS AND SUMMARY.

The properties of the oxidase, peroxidase, catalase, and amylase of cabbage, carrot, yellow and white turnip, tomato, and potato juices were studied at different hydrogen ion concentrations. Marked changes in the enzyme contents and properties were found on dehydrating these vegetables, air blast dehydration producing considerably greater changes than vacuum dehydration.

In considering these enzyme results, it may be pointed out that the state of ripeness and the age of the vegetable undoubtedly influence the activities. More complete series of results taking these factors into account would give results of interest. In this work, ordinary fresh vegetables, as purchased in the open market, were used.

With regard to a practical question of dehydration, the air-dehydrated potatoes prepared commercially are boiled before dehydration. Those used in this investigation were not boiled. The vacuum-dehydrated potatoes were obtained white in color, the air-dehydrated grey to black. On treating the vacuum-dehydrated potatoes with water of the hydrogen ion concentration 10^{-5} N or more, no darkening was noticeable, with water of the hydrogen ion concentration 10^{-6} N or less, comparatively rapid blackening occurred. The potato in all probability has a natural hydrogen ion concentration of 10^{-6} to 10^{-7} N.[10] If this could be increased to 10^{-5} N before dehydration, then even air dehydration should give a colorless product. Treatment with dilute acid solution before dehydration may accomplish this.

Since heat and time are probably the determining factors in bringing about the changes in enzyme actions, it would appear that the shorter the period of time in which heat is applied, the smaller will be the changes. Vacuum dehydration can be carried out in 8 to 12 hours or less; air blast dehydration requires longer periods of time. This may be one of the reasons, in addition to the possible changes brought about by the oxygen of the air, for the smaller changes in the vacuum dehydration products as compared with the air blast dehydration products.

[10] Cohn, E. J., and Gross, J. Data to be published later in *J. Gen. Physiol.*

This point may also be mentioned in connection with the food hormones. It is possible that cooking at a high temperature for a short period of time[11] will not cause the destruction of these properties, that heating in a current of air for a long time will destroy them, and that heating for an intermediate period of time in the absence of air may do so partially.

The differences in the titration curves between the juices from the fresh and the dehydrated vegetables indicate also that some change takes place on dehydration, greater in air blast dehydration than in vacuum dehydration.

With regard to the relation between the chemical properties or structures upon which the actions of enzymes and of food hormones depend, one significant fact is that enzymes are inactivated by heating in solution for a short time, while food hormones as a rule are not. On the other hand, the partial or complete inactivation of both in many cases in dehydration indicates a possible similarity. No evidence is available as to the nature of the chemical groupings responsible for these actions. All that can be said at present is that the enzyme property is destroyed more readily than the food hormone properties. The enzyme property is perhaps the most sensitive index of change taking place in material obtained from living matter susceptible to tests by simple chemical means. A change in the enzyme properties need not be accompanied necessarily by a change in the properties of the food hormones.

[11] Delf, E. M., *Biochem. J.*, 1918, xii, 416.

ACIDIMETRIC TITRATION OF GRAIN EXTRACTS AND AMINO-ACIDS IN THE PRESENCE OF ALCOHOL.*

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The observation described here was first made in December, 1915, during a study of the sugar content of several aqueous extracts of oats. A number of finely ground samples of oats had been extracted with ice water in a cold storage room for 1 hour and the filtered extracts were neutralized with 0.1 N sodium hydroxide, phenolphthalein being used as an indicator. A large excess of neutral ethyl alcohol was then added in order to precipitate dextrins and other gummy substances. I noticed that if alcohol was added to a given aliquot of the cold extract before titration considerably more alkali was required for complete neutralization than when no alcohol was present. This apparent change in acidity took place immediately after adding the alcohol, notwithstanding the low temperature of the two liquids and of their mixture. Extracts of other cereals behaved in the same way. In most cases a precipitate was formed upon the addition of alcohol. When the aqueous extract to which alcohol had been added was heated without previous neutralization until all the alcohol was driven off, the precipitate did not redissolve upon the addition of distilled water, but the acidity of the resulting solution was the same as that of the original aqueous extract. This shows that the substances which are precipitated or coagulated by the alcohol probably bear no direct relation to the shift in acidity.

It seemed important to investigate the influence which different concentrations of alcohol might have on the titration value

*An abstract of this paper was read before the 33rd annual convention of the Association of Official Agricultural Chemists at Washington, D. C., in November, 1916.

of the mixture. I found for oats that the titration values do not increase in linear proportion to the amount of alcohol which was present, but that the rise is slow in the beginning, then gradually becomes more rapid, and finally the values remain constant. If plotted on paper, they form an S-shaped curve (Fig. 1).

If mixtures of oats or corn extracts with ethyl alcohol were allowed to stand for several days at either ordinary or cold storage temperatures, without being neutralized, their acidity remained practically unchanged; if neutralized, more acid was formed. Moreover, a greater amount of acid was produced in cases when the mixtures had been neutralized at once than when they had stood for several days before being neutralized. Possibly the formation of fresh acid is due to an enzyme which is destroyed during prolonged contact with alcohol. It may be assumed that the enzyme possesses acid properties. This would explain the fact that in the presence of alcohol the acidity values of unneutralized extracts are a trifle lower after several days standing than at the start (see Tables II and III).

Experiment 1.—18 gm. of ground oats were extracted with 150 cc. of ice water for 70 minutes. Of the filtered extract, 25 cc. aliquots were pipetted into two Jena beakers. The one aliquot was neutralized with 0.1 N NaOH, with phenolphthalein as indicator. 0.68 cc. was required to produce a pink color. To both aliquots 35 cc. of neutral absolute alcohol were now added. A white precipitate was formed in each one, and in the aliquot which had already been neutralized the pink color disappeared. An additional 0.82 cc. of 0.1 N alkali was required to produce again the pink coloration. The amount of alkali required to neutralize the 25 cc. of aqueous oat extract to which 35 cc. of neutral alcohol had been added was, therefore, more than twice the amount required when no alcohol was present.

The other beaker, the contents of which had not been neutralized, was placed on the steam bath until all the alcohol had been driven off. Care had to be exercised not to carry the evaporation too far, since otherwise decomposition takes place, which is accompanied by a rise in acidity. After adding about 20 cc. of distilled water, the liquid required 0.69 cc. of 0.1 N NaOH for neutralization. The white precipitate had not redissolved.

With 18 gm. of corn-meal, if extracted in exactly the same manner, similar results were obtained, the values found being as follows:

25 cc. of extract required directly.....	0.87 cc. of 0.1 N NaOH
With 35 cc. of alcohol added there were required an additional.....	0.73 " " 0.1 N NaOH
Total.....	1.60 " " 0.1 N NaOH

The evaporated aliquot, after adding water, required..... 0.86 cc. of 0.1 N NaOH

Experiment 2.—Measured amounts of neutral absolute alcohol were placed in dry 100 cc. Erlenmeyer flasks and the latter closed with rubber stoppers. 156 gm. of ground oats (containing 5.6 per cent of moisture) were extracted with 1,200 cc. of ice water for 70 minutes, with occasional stirring. The infusion was poured upon folded filters, and the filtrates were united and well mixed. 20 cc. portions of this filtrate were measured from a burette into each of the Erlenmeyer flasks containing different amounts of alcohol. Each mixture was made up in duplicate. The extraction of the oats and the measuring off of the 20 cc. aliquots were carried out in a cold storage compartment having a temperature of 1–2°C. The various alcohol-extract mixtures were then titrated with 0.1 N NaOH, phenolphthalein being used as indicator.[1] The titration values are given in Table I.

In Fig. 1 are shown the S-shaped curves which represent the changes in acidity with varying concentrations of alcohol. In the lower curve the strength of the alcohol is expressed in volumetric per cent, while in the upper the abscissæ represent the number of cc. of alcohol which in each case was mixed with 20 cc. of extract. The curves seem to show a second short rise between the alcohol concentrations of 60 and 65 per cent; that is, just before reaching the final maximum. The experiment was repeated twice and this second rise was observed in each case.

Experiment 3.—To 20 cc. aliquots of ice water extracts of oats and cornmeal were added measured amounts of neutral alcohol. The mixtures were made up in triplicate. The one set was neutralized at once, the

[1] McCoy (*Am. Chem. J.*, 1904, xxxi, 508) noted that the addition of alcohol reduces the color intensity of alkaline solutions of phenolphthalein. This phenomenon, for which he offered no satisfactory explanation, is doubtless responsible to a slight extent for the rise in acidity reported in this paper. However, since not more than five drops of a 0.5 per cent solution of phenolphthalein (previously neutralized to pale red) were used for any of my titrations, the shift due to the indicator may be considered as negligible. Morey (*J. Am. Chem. Soc.*, 1912, xxxiv, 1031), in titrating benzoic acid in strong alcohol with three drops of 1 per cent phenolphthalein as indicator, required only from 0.06 to 0.08 cc. of 0.1 N alkali in his blanks. I have myself titrated a number of weak acid solutions, *e.g.* of acetic, tartaric, and lactic acid, in the same manner as the grain extracts without obtaining an appreciable shift of acidity in the presence of alcohol.

TABLE I.

Relation Between Concentration of Alcohol and Neutrality Point (Phenolphthalein) of an Ice Water Extract of Oats.

Alcohol added to 20 cc. of extract.	Alcohol content of mixture.	0.1 N NaOH required.	Average.
cc.	*per cent*	*cc.*	*cc.*
0	0	0.60 0.60	0.60
1	4.8	0.62 0.62	0.62
3	13.0	0.66 0.66	0.66
5	20.0	0.71 0.73	0.72
7	25.9	0.83 0.83	0.83
9	31.0	0.94 0.95	0.945
11	35.5	1.05 1.05	1.05
13	39.5	1.11 1.13	1.12
15	42.9	1.18 1.20	1.19
17	46.0	1.23 1.25	1.24
19	48.7	1.28 1.30	1.29
21	51.2	1.32 1.32	1.32
23	53.5	1.34 1.36	1.35

TABLE I—*Concluded.*

Alcohol added to 20 cc. of extract.	Alcohol content of mixture.	0.1 N NaOH required.	Average.
cc.	*per cent*	*cc.*	*cc.*
30	60.0	1.39 1.41	1.40
32	61.5	1.41 1.41	1.41
34	63.0	1.43 1.47	1.45
36	64.3	1.44 1.46	1.45
38	65.5	1.47 1.47	1.47
40	66.7	1.47 1.45	1.46
42	67.7	1.47 1.48	1.475

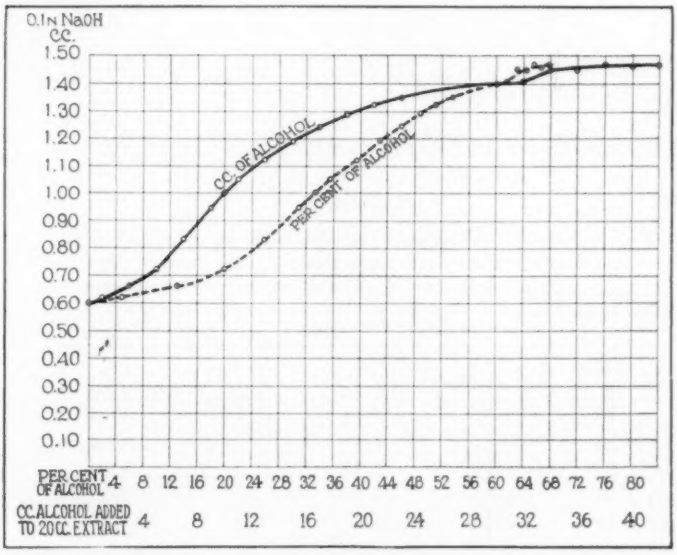

FIG. 1. Relation between concentration of alcohol and neutrality point (phenolphthalein) of an ice water extract of oats.

TABLE II.

Effect of Keeping Alcohol-Extract Mixtures for Some Time before and after Neutralizing.

Alcohol added to 20 cc. of extract.	Titrated at once.		Titrated after 24 hrs. at 1°C.		Titrated after 48 hrs. at 1°C.		Titrated after 48 hrs. at 25°C.	
	Original titration.	Increment 2 days later.	Original titration.	Increment 2 days later.	Original titration.	Increment 2 days later.	Original titration.	Increment 1 day later.
	0.1 N NaOH required for neutralization.							
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Oats.								
0	0.69	0.20	0.69	0.20				
5	0.87	0.24	0.82	0.17				
10	1.12	0.40	1.07	0.11			1.15	0.09
15	1.28	0.45	1.29	0.11			1.28	0.11
20	1.42	0.65	1.37	0.13			1.34	0.10
25	1.48	0.61	1.41	0.24			1.38	0.12
Corn-meal.								
0	0.88	0.25			0.88	0.25		
5	1.09	0.18			1.05	0.11		
10	1.30	0.22			1.20	0.13	1.20	0.13
15	1.46	0.23			1.30	0.18	1.33	0.11
20	1.55	0.31			1.39	0.21	1.40	0.15
25	1.65	0.32			1.44	0.31	1.50	0.12

With another oat extract, the results were as follows:

TABLE III.

Effect of Keeping Alcohol-Extract Mixtures before Neutralizing.

Alcohol added to 30 cc. of oat extract.	0.1 N NaOH required for neutralization.	
	Titrated at once.	Titrated after 72 hrs. at 1°C.
cc.	cc.	cc.
12	1.15	1.10
16	1.21	1.23
20	1.31	1.30
25	1.35	1.34
30	1.39	1.35
35	1.47	1.43

second was placed in the cold storage room in stoppered flasks before being neutralized. The third set, containing only four mixtures, was kept at room temperature. The time during which the different mixtures were kept, before being neutralized, is indicated in Tables II and III.

It is seen from Tables II and III that the acidity of these extracts to which alcohol had been added does not change markedly on standing, and that whatever slight changes may take place consist in a destruction of acid substances.

After the first neutralization, the various mixtures of alcohol and oat extract formed more acid. The amounts of this secondary acid formation were determined by a new titration after standing. They are designated as increments in Table II. It is seen that if the first neutralization has taken place soon after adding the alcohol to the extract, the secondary acid formation was much stronger than if the liquids had not been neutralized at once, a fact which may be due to a gradual disintegration of acid-forming ferments.

Experiment 4.—Samples of 15 gm. each of the following ground materials were extracted with 150 cc. of ice water for 70 minutes: polished rice, pearl barley, whole wheat, whole rye, white kidney bean. The extracts were filtered and duplicate aliquots of 30 cc. each (corresponding to 3 gm. of material) pipetted into 100 cc. Erlenmeyer flasks. To the one aliquot of each, 30 cc. of neutral absolute alcohol were added, while the other aliquot was titrated directly, using phenolphthalein as indicator. The results are shown in Table IV.

TABLE IV.

Shift in Acidity of Ice Water Extractions of Various Seeds Caused by Addition of Alcohol.

	0.1 N NaOH neutralizing 30 cc. of extract (= 3.0 gm. of substance).	
	Without alcohol.	After adding 30 cc. of neutral alcohol.
	cc.	cc.
Polished rice	0.12	0.25
Pearl barley	0.71	1.01
Whole wheat	0.46	1.07
" rye	1.57	2.88
White kidney bean	3.10	5.68

It is seen from Table IV that the phenomenon reported above is not limited to cereals but that it also occurs with extracts of other seeds. However, no secondary acid formation took place in the case of the bean extract, while all grain extracts, after being neutralized, formed fresh acid if allowed to stand for a few hours.

Inasmuch as the facts observed seemed to have some bearing on the interpretation of titration results which have been sometimes obtained in the presence of alcohol,[2] a brief attempt was made to determine the nature of those grain constituents which are responsible for this remarkable shift in acidity. It was soon found that the phenomenon is probably attributable to amino compounds present in the extracts, since, as demonstrated in the following experiments, solutions of these substances show the same behavior.

Dilute aqueous solutions (approximately 0.12 per cent) of tyrosine and leucine were prepared. To 25 cc. aliquots of these solutions 25 cc. of neutral alcohol were added, and in a control experiment 25 cc. of distilled water. On neutralizing these liquids, with phenolphthalein as indicator, the following results were obtained:

	0.1 N NaOH required	
	Without alcohol.	In 50 per cent alcohol.
	cc.	*cc.*
Leucine	0.08	2.24
Tyrosine	0.44	2.50

Therefore, with these two monoamino-acids, a strong shift in acidity actually took place upon adding ethyl alcohol.

The matter was further studied with solutions of glycocoll, alanine, aspartic acid, and hippuric acid, all of Kahlbaum's manufacture. The results are set forth in Table V, and graphically in Fig. 2.

[2] A paper on this subject by myself will appear shortly in the *Journal of Agricultural Research*. The recent findings of Ferraro (*Boll. chim. farm.*, 1915, liv, 257, abstracted in *Chem. Abstr.*, 1916, x, 3042) harmonize with my own observations.

TABLE V.

Change in Acidity of Amino-Acid Solutions, Caused by Addition of Alcohol.

Alcohol added before titration to 10 cc. of aqueous solution of acid.	0.05 N NaOH required to produce pink coloration of phenolphthalein.			
	Glycocoll.	Alanine.	Aspartic acid.	Hippuric acid.
cc.	*cc.*	*cc.*	*cc.*	*cc.*
0.0	0.40	0.18	2.50	1.41
1.5	0.55			
2.0		0.50		
2.5	0.85			
3.0				1.41
4.0		1.30		
5.0	2.00		2.85	
6.0		1.63		1.41
7.5	3.00			
8.0		2.38		
10.0	3.70	2.90	3.35	1.41
12.5	3.90	3.30		
15.0	4.40	3.60	3.65	1.41
17.5	4.40	4.00		
20.0		4.20	3.80	
25.0		4.45	3.85	

The solutions of the two monoamino-acids were of approximately the same molecular strength, containing per 100 cc. respectively, 0.198 gm. of glycocoll, and 0.235 gm. of alanine. The acidity of the latter is seen to rise more slowly than that of the former, but both appear to reach the same maximum.

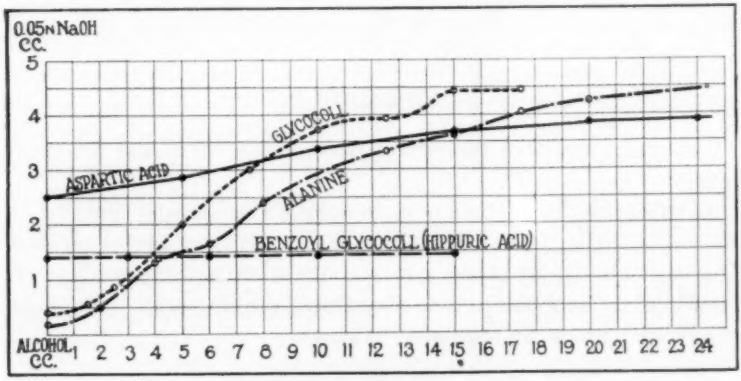

FIG. 2.

With the dibasic aspartic acid, of which an 0.18 per cent solution was used, the increases due to alcohol, although quite measurable, are much less pronounced than with the monobasic acids.

Unfortunately arginine or histidine could not be tested for want of material. These two amino-acids, which in aqueous solution possess an alkaline reaction, may be expected to react acid to phenolphthalein in the presence of sufficient alcohol.

Hippuric acid, which is not an amino-acid, but which may be obtained synthetically from glycocoll, was included in this study merely to show that the phenomenon in question is very likely confined to compounds containing the NH_2 group. The solution used contained 0.125 gm. of hippuric acid per 100 cc. No rise in acidity was observed upon addition of alcohol.

The phenomenon described is possibly due to a simple substitution reaction with the hydrated form in which, according to Robertson,[3] amino-acids exist in aqueous solution, and to which he attributes their amphotheric character. This reaction could be expressed in the case of glycocoll, by the equation

$$CH_2\begin{matrix}NH_3OH\\COOH\end{matrix} + C_2H_5OH \rightarrow CH_2\begin{matrix}NH_3\cdot O\cdot C_2H_5\\COOH\end{matrix} + H_2O$$

and the elimination of the hydroxyl group would account for the loss of the amphotheric character of these bodies in the presence of alcohol.

The observation may have practical importance, inasmuch as under certain conditions it may furnish a convenient measure for the rate of decomposition of the proteins contained in a given solution.

SUMMARY.

The main result of this study may be stated in the following terms.

Amino-acids, which in aqueous solution are nearly neutral to phenolphthalein, react distinctly acid in the presence of alcohol. This fact should be taken into account when making acidimetric titrations in alcoholic liquids containing amino compounds, such as various animal or vegetable extracts.

[3] Robertson, T. B., *Ergebn. Physiol.*, 1910, x, 216.

PREPARATION OF RHAMNOSE.

By E. P. CLARK.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 29, 1919.)

Methods for the preparation of rhamnose were published by Kruis[1] and by Rayman.[2] These authors found that commercial extract of black oak bark known as "quercitron extract" was a convenient material for the preparation of this sugar. The process employed by these authors was as follows.

Quercitron extract was hydrolyzed by boiling with dilute sulfuric acid for ½ hour. This solution was filtered and neutralized with calcium or barium carbonate. The solution after filtering was concentrated to a thick syrup and allowed to crystallize.

When this process was applied to material obtainable at the present time the result was very unsatisfactory. However it was found possible to obtain quite satisfactory yields of rhamnose by a modification of the original method. The modified process is as follows.

2 kilos of the liquid quercitron extract,[3] which has the consistency of a thick syrup, is dissolved in enough hot water to make 5½ liters. It is brought to a boil and 180 cc. of concentrated sulfuric acid dissolved in 320 cc. of water are gradually added, thus making the concentration of the acid 3 per cent by volume. The mixture is gently boiled for 30 minutes, then poured into a crock jar or enameled kettle, and quickly cooled.

The liquid after being separated from the insoluble material is neutralized with barium hydroxide. The barium sulfate is filtered off, the filtrate evaporated to a thin syrup (approxi-

[1] Kruis, K., Sitzungs berichte der Böhmischen Gesellschaft der Wissenschaften, Prague, 1877-78, 157.

[2] Rayman, B., *Bull. Soc. Chim.*, 1887, xlvii, 668.

[3] The material used in this work was obtained from A. Klipstein and Co. of New York.

mately 350 cc.), and 8 volumes of 95 per cent alcohol are slowly added with constant stirring. The heavy precipitate formed is filtered off and sucked dry on a Büchner funnel. The resulting filtrate is evaporated under diminished pressure to a thick syrup, dissolved in 1 liter of 95 per cent alcohol, and to the solution $2\frac{1}{2}$ liters of ether are added. A gummy substance is precipitated, which, after decantation of the supernatant liquid, is dissolved in 50 cc. of water and enough alcohol to make 1 liter, the alcohol being slowly added and thoroughly mixed with the syrup. The solution is precipitated again with $2\frac{1}{2}$ liters of ether. The liquid is decanted from the precipitate and the two etherial extracts mixed and allowed to stand over night during which time they become clear. The clear solution is poured off from the gummy substance which settled out, the ether is recovered by distilling on a steam bath, and the remaining alcoholic solution is evaporated under diminished pressure to a thick syrup. This is removed from the flask with an equal volume of alcohol. To the solution ether is then added. Upon scratching the flask, or quicker by seeding, the rhamnose separates out, requiring generally a day for complete crystallization. The sugar is filtered off as dry as possible on a Büchner funnel, washed first with a mixture of 1 part ether and 2 parts alcohol, then with a 1:1 alcohol and ether mixture, and finally with ether.

The yield is from 50 to 55 gm. of white sugar. It may be recrystallized from water or alcohol in the usual way.

THE STABILITY OF LACTALBUMIN TOWARD HEAT.

By A. D. EMMETT and G. O. LUROS.

(*From the Research Laboratory, Parke, Davis and Company, Detroit.*)

(Received for publication, April 21, 1919.)

Since there seems to be some specific and characteristic difference between the growth-promoting value of casein and lactalbumin, which is dependent upon certain dietary conditions as shown by comparing the results of Osborne and Mendel,[1] McCollum, Simmonds, and Parsons,[2] and Emmett and Luros,[3] we have made a further study of this subject by undertaking to ascertain whether heat will affect the nutritive value of lactalbumin to the extent that McCollum and Davis claim that it destroys the growth-promoting value of casein.

McCollum and Davis[4] reported that, by long continued heating of milk at 90–100°, the nutritive value of the casein was impaired; that, when casein was heated in an autoclave for 1 hour at 15 pounds pressure, it lost its "biological value as a complete protein," due to some of the amino-acids being broken down; that wet milk powder heated for a time in a double boiler or for 1 hour in the autoclave at 15 pounds pressure was deficient in promoting growth; that evaporated milk whey was not affected by heating in the autoclave; and that unrefined lactose retained as much of the adhering water-soluble B accessory after heating as before. In view of these findings, McCollum and Davis concluded that it would not be practicable to purify casein by washing it with hot alcohol as Funk and Macallum[5] had done, and so proposed a method of purification which did away with the use of heat entirely. Funk and Macallum in turn prepared casein by these two methods and found that there was no difference be-

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351; *ibid.*, 1916, xxvi, 1.

[2] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 287.

[3] Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 147.

[4] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231, 247.

[5] Funk, C., and Macallum, A. B., *Z. physiol. Chem.*, 1914, xcii, 17; *J. Biol. Chem.*, 1916, xxvii, 51.

tween them in respect to their growth-promoting value. Upon heating the casein in the autoclave, however, they obtained evidence which confirmed McCollum and Davis as to a loss in the biological value of casein. They attributed the change in the nutritive value of the protein to a destruction of the antiscorbutic vitamine, and not to a breaking down of the protein. Hogan[6] studied the effect of heat on the growth-promoting value of corn, egg albumin, and casein. His data indicate that when corn was heated in the autoclave, no growth resulted, while excellent results were obtained with unheated corn. Egg albumin, heated in the autoclave, and then combined with protein-free milk, butter, starch, and agar so as to make a 9 per cent protein diet, was very slightly, if at all, impaired. Upon heating casein in an autoclave under 15, 30, and 45 pounds pressure, respectively, for 2 hours, and making up three corresponding rations with protein-free milk, butter fat, starch, and agar, and comparing each with a ration containing unheated casein, Hogan obtained results which indicated that casein was not affected by heat. His conclusions were that heat does not lower the nutritive value of the casein, but that one or more of the food accessories may be affected.

In the series of tests reported here on the effect of heat upon the nutritive value of lactalbumin, we subjected this protein to different temperatures and then incorporated it in the basal diet. The control supply of lactalbumin was dried *in vacuo* at 55–60° (Chart 1). Portions of this lot were then heated as follows: (*a*) in the air oven at 90–100° for 16 to 18 hours (Chart 2); (*b*) in the autoclave at 15 pounds pressure for 2 hours (Charts 3, 4, and 5); and (*c*) in the autoclave at 15 pounds pressure for 6 hours (Chart 6). The basal diet was composed of protein-free milk 28 per cent; butter fat 5, 18, or 28 per cent; lard none or 10 per cent; and starch to make up the balance after adding the protein. In each case, 10 per cent of lactalbumin protein was employed, this being based upon the nitrogen content of each of the proteins.

The protocols accompanying the charts give the details of the different tests. It will be seen, from the curves showing the rate of growth for the different groups of rats, that heating the lactalbumin had no effect upon its growth-promoting value.

In view of this fact, it is of interest to compare these results with our former conclusions, that lactalbumin is a complete protein for growth,[3] and to correlate the evidence that we put

[6] Hogan, A. G., *J. Biol. Chem.*, 1916, xxvii, 193; *ibid.*, 1917, xxx, 115

forth to show that the difference between lactalbumin and casein may be based on the vitamine hypothesis.

Studying the basal diets of McCollum and Davis, Funk and Macallum, and Hogan, it will be seen that their experimental rations differed in two essential points: first, in respect to the amount of butter fat used, and second, in respect to the source of water-soluble vitamines employed. Thus, McCollum and Davis used 5 per cent butter fat and obtained their water-soluble vitamines from powdered milk, milk whey, wheat germ, or lactose. Funk and Macallum employed no butter fat but obtained the fat-soluble A accessory from dried yeast which also furnished the water-soluble B. Orange juice was also used. Hogan incorporated 30 per cent of butter fat in his rations, and for the water-soluble vitamines he used either corn or protein-free milk.

From the standpoint of the amount of butter fat, it might be said that Hogan obtained normal growth with heated casein because he used 30 per cent of butter fat, while McCollum and Davis secured poor results on account of having only 5 per cent of the fat present. It is easy to conceive that the larger amount of butter fat might have altered the palatability of the ration so that the food intake was modified and thereby produced a difference in gain in weight; or it might be that this diet was more easily assimilated. Osborne and Mendel[7] and Maignon[8] found that the availability of protein varied directly with the amount of fat present. The data given in Charts 3, 4, and 5, where 18, 5, and 28 per cent, respectively, of butter fat was mixed with the 2 hour autoclaved lactalbumin, indicate clearly that the amount of butter fat had no effect upon the rate of growth. One group of rats grew as well as another and the body condition of each was equally good. This is brought out more clearly perhaps when the food intake is considered. Table I shows that the group consuming the low fat diet ate more food than did the one on the high fat ration. In other words, the low fat group consumed much more protein, and if the heated lactalbumin had been toxic, we would not have obtained results which were equal to those made by the high fat group where less protein was ingested. Further, in comparing the food intake for the heated and un-

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.
[8] Maignon, F., *Compt. rend. Acad.*, 1918, clxvii, 172.

heated lactalbumin rations with 18 per cent butter fat and 10 per cent lard, the differences were so slight that it is evident that the heated protein was not toxic. These findings suggest that the percentage of butter fat in the ration had no special function in making the heated lactalbumin more available than the unheated protein, and it is more than likely that it had no influence with the heated casein which Hogan used.

With regard to the water-soluble vitamines, in the case of McCollum and Davis' report, it so happened that in their various rations which contained milk or its components, they had present, when they obtained good growth, some constituents of the milk which had *not* been heated. Thus, with the heated casein they had unheated milk whey or lactose while with the unheated

TABLE I.

Average Values for Each Group for Five Weeks.

Lactalbumin.	Fat.			Gain per day.	Food intake per day.	Gain per gm. of food.
	Butter fat.	Lard.	Total.			
	per cent	*per cent*	*per cent*	*gm.*	*gm.*	*per cent*
Unheated.....	18	10	28	1.56	6.6	23.6
6 hour autoclave.....	18	10	28	1.50	7.8	19.2
2 " "	18	10	28	1.34	6.5	20.9
2 " "	28	—	28	1.72	7.7	22.8
2 " "	5	—	5	1.60	12.9	12.4

casein they used heated whey or lactose. In other words, their combination of milk food substances was such that there was a good possibility that the unheated constituent carried an accessory (which we have already suggested as being able to be adsorbed by lactose and possibly by casein) and that this was responsible for the growth on the rations. To be sure the authors cover this point in part by showing that the wheat embryo can be heated for 1 hour in the autoclave at 15 pounds pressure and then when it is added to a diet of polished rice and butter fat, it is still able to supply the water-soluble B vitamine. However, from some of our results which are now in preparation for publication, we have evidence to show that heat will partially destroy a particular vitamine (other than the water-soluble B) that relates to growth, and that the rate of growth will depend largely

upon the amount of the substance present and also upon the degree of heating. That is, if the minimum amount or slightly more of the unheated material, which carries the vitamine, is present, normal growth will occur, but heating this vitamine will cause poor growth or no growth at all. If, however, there is an excess of the vitamine present in the unheated food, the effect of heating it will not be so marked.

Funk and Macallum obtained poor growth with heated casein even when dried yeast was in the ration, but when 1 cc. of orange juice was added each day they obtained good results. This stimulation of growth was attributed to the antiscorbutic vitamine in the juice. Hogan supplemented each of his casein rations with protein-free milk just as we did with lactalbumin. Protein-free milk contains 80 per cent of lactose and we[3] have shown that lactose has some peculiar biological property of stimulating growth which is due either to its ability to overcome toxicity or else to a vitamine (other than the water-soluble B) which it appears to adsorb. Therefore, it may be that the lactose in the protein-free milk carried enough of the accessory or vitamine to effect normal growth with casein in Hogan's experiments. Chart 7 illustrates how the addition of lactose, as a partial substitute for starch in an otherwise complete diet, stimulated growth.

Our inference would be, therefore, that this accessory or vitamine was the factor that brought about the difference between the values of heated casein of McCollum and Davis on the one hand, and of Hogan on the other. This vitamine is different from the so called water-soluble B which is stable toward heat. Whether it is the same as the antiscorbutic vitamine, as suggested by Funk and Macallum, is being studied. Pitz[9] claims that lactose used as a supplement to certain foods will prevent the onset of scurvy for 20 weeks. Cohen and Mendel[10] found that with very highly purified lactose scurvy was not retarded. In some preliminary experiments, we have found that our purified lactose was not efficacious in curing scurvy in guinea pigs in the advanced stage. Further study on the specific function of lactose is under way.

[9] Pitz, W., *J. Biol. Chem.*, 1918, xxxiii, 471.

[10] Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

CONCLUSIONS.

From the results of the effect of heat on lactalbumin the data indicate: (1) that high temperatures do not influence the growth-promoting value of lactalbumin when dried *in vacuo* at 55°, or heated in an air oven at 90–100° for 16 hours, or in an autoclave at 15 pounds pressure for 2 and 6 hours, respectively; (2) that the amount of butter fat, whether 5, 18, or 28 per cent when used with a 2 hour autoclaved lactalbumin, has no influence on the rate of growth; (3) that heated lactalbumin is not toxic for young growing rats; (4) that our previous conclusion regarding the excellent growth-promoting value of lactalbumin is further substantiated on the hypothesis that there is a vitamine factor involved which is different from the water-soluble B.

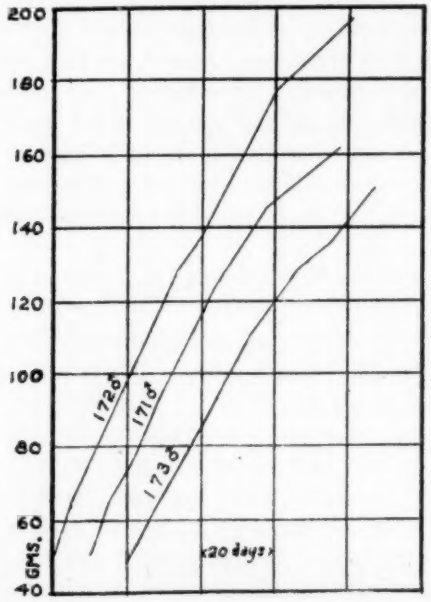

CHART 1. The rats in this group were fed a ration with 10 per cent lard and 18 per cent butter fat, and 10 per cent of lactalbumin protein which had been thoroughly purified by washing with hot water, then digesting a number of times with hot alcohol, and finally washing with ether. This lactalbumin was then dried *in vacuo* at 55–60° for 12 hours. Normal growth resulted.

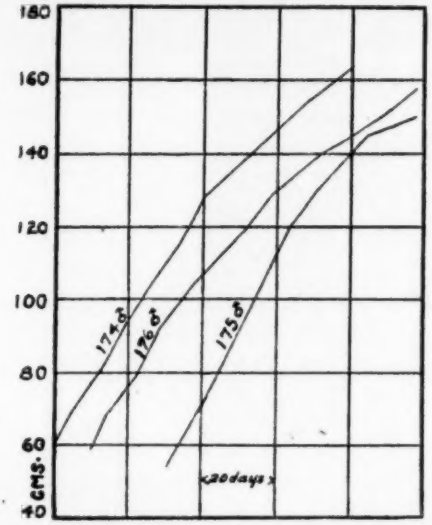

CHART 2. The diet for these rats was the same as that for the animals in Chart 1, except that the lactalbumin was heated at 90–100° for 16 to 18 hours in an air oven. There was very little difference in the rate of growth between this group and that fed the unheated lactalbumin.

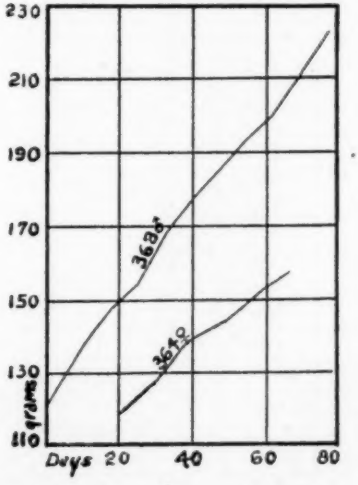

CHART 3. The diet differed from the control ration (Chart 1) in having 10 per cent of protein from lactalbumin which had been heated in the autoclave for 2 hours at 15 pounds pressure.

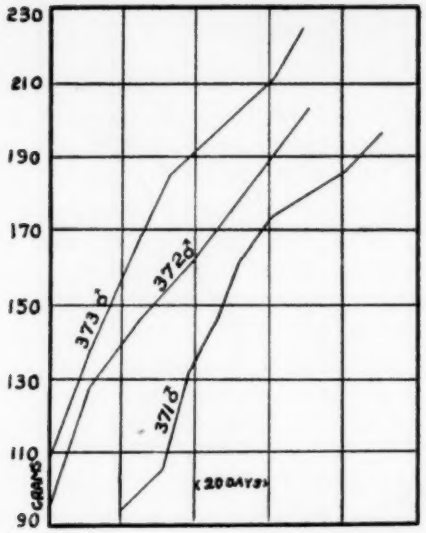

CHART 4. The ration was the same as that fed to the rats for Chart 3, except that only 5 per cent of butter fat and no lard were used.

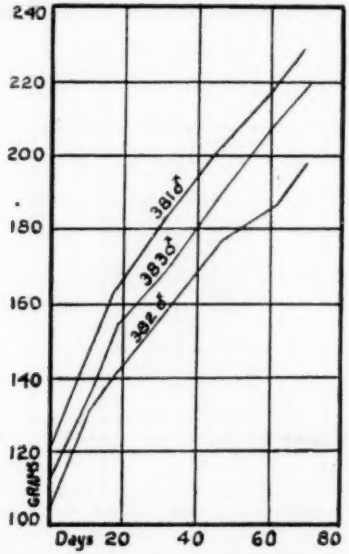

CHART 5. The ration used for these rats was the same as that fed to the group represented in Charts 3 and 4, except for the increase in butter fat to 28 per cent.

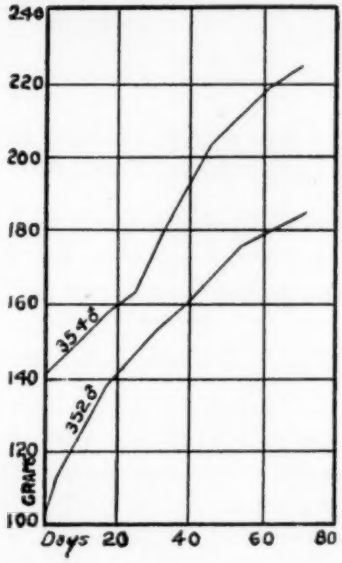

CHART 6. The ration differed from the control diet (Chart 1) and those for Charts 2 and 3, in having 10 per cent protein from lactalbumin which had been heated in the autoclave for 6 hours at 15 pounds pressure.

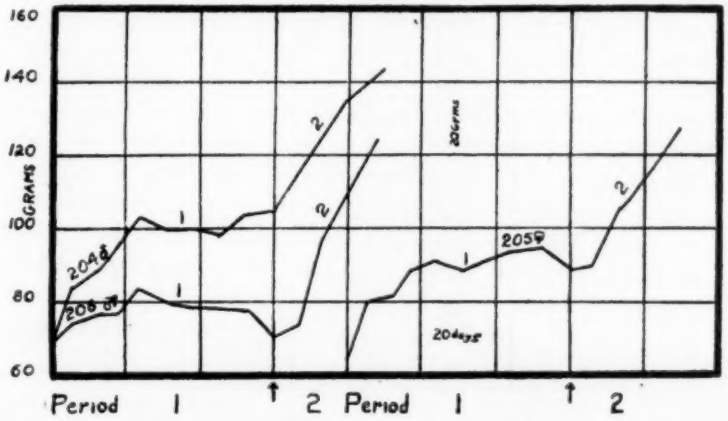

CHART 7. The diet for Period 1 was composed of unheated lactalbumin protein, 10 per cent; butter fat, 18 per cent; lard, 10 per cent; hot alcohol extract of wheat germ, 5 per cent; and starch to make up the balance. In Period 2, part of the starch was replaced by 24.6 per cent of purified lactose; otherwise the diet was the same as in Period 1. This shows that the addition of lactose stimulated growth.

MAINTENANCE AND PRODUCTION VALUE OF SOME PROTEIN MIXTURES.*

By E. B. HART and H. STEENBOCK.

With the Cooperation of F. Letcher.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

(Received for publication, April 8, 1919.)

It is too well known to require further amplification that the efficiency for growth of a protein mixture will depend upon the qualitative and quantitative make-up of its amino-acid content. The supplementing effect of specific amino-acids for certain definite proteins has been especially studied by Osborne and Mendel,[1] but up to the present time very little data have been accumulated on the efficiency for growth of practical protein mixtures in common use, both in human and in animal nutrition. Some earlier studies in this laboratory by McCollum[2] disclosed the relatively low efficiency for growth of the cereal grain proteins when fed alone or in mixtures and a much higher utilization of casein and milk proteins.

In addition to the above work data have been collected[3] which showed considerable variation in the utilization of protein mixtures from plant sources for milk production which, in its physiological demands, may be correctly compared with growth phenomena. All these studies have a decided practical bearing as they show how a greater utilization of a poor protein mixture can be accomplished by adding to it some single protein or a mixture of proteins with proper supplementing qualities.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325; 1916, xxv, 1; 1915, xx, 351; 1917, xxix, 69.

[2] McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

[3] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239; 1916, xxvi, 457; 1917, xxxi, 445; 1918, xxxv, 367.

In popular discussions of nutrition problems it is all too common practice at the present time to condemn the cereal grains or other seed products in respect to their protein efficiency. Such condemnation would be warranted if a single cereal grain or a mixture of cereal grains served as the sole diet, but such is rarely the case. What is needed at the present time is definite knowledge as to what products and in what amounts efficient supplementary effects can be secured so that a distinct improvement in the utilization of the proteins of cereals may be brought about. Further, the production value of protein mixtures should be given just as definite an expression as has been given to the net energy values of feeds. True, the production value of proteins, numerically expressed, will be modified by the nature of the protein mixture, by the proportions of proteins from varying sources in the mixture, and by the plane of protein fed. All these factors complicate the problem. For example, the net energy value of 100 lbs. of corn-meal when fed to ruminants is 88.8 therms, which becomes a fixed and definite figure under any conditions of feeding corn-meal to a ruminant. But the production value of the proteins of corn-meal when fed alone to swine is approximately 25; that is from 100 lbs. of corn proteins 25 lbs. can be retained for growth, in addition to maintenance, and this figure 25 would be materially increased if the corn proteins were fed with milk proteins, but with the protein intake at the same level as when corn proteins were fed alone. Further, the figure would be increased if in feeding corn proteins the plane of protein intake were lowered, and *vice versa*. These facts make the problem of determining the production value of proteins a somewhat complex one.

EXPERIMENTAL.

In the use of natural materials such as milk as a supplement to grains there is a marked increase in the nitrogen retention of the mixture by a growing animal. This is interpreted as an improvement in the character of the amino-acid mixture ingested. In the present state of our knowledge an experiment on growth with a grain plus milk is open to the criticism that a grain ration may have been greatly improved by the milk supplement through the addition of extra mineral matter, fat-soluble vitamine, etc.,

which are recognized as grain deficiencies. It is yet to be determined whether the inferior showing for growth made by the proteins of cereal grains when fed alone is to be attributed to their deficiency in other factors of nutrition such as the vitamines and mineral matter. To determine the validity of this criticism and to standardize our experimental procedure, pigs of 50 to 75 lbs. in weight have been fed grain mixtures alone and grain mixtures to which were added the most likely deficiencies of the grains other than poor proteins; namely, butter fat as a source of the fat-soluble vitamine, and mineral matter.

In all these test experiments, as well as those which followed, the procedure has been as follows: Vigorous pigs of 50 to 75 lbs. in weight, selected from the Station herd, were given a preliminary starch feeding period of 4 days. At the end of this time they were placed in metabolism cages by which a balance of intake and outgo of nitrogen was determined over a period of 24 days.

There is positive evidence, as shown in experiments on Pigs 30 to 33, Table I, that over a period of 24 days accurate data on the protein efficiency of grains can be secured without vitamine and mineral additions. Butter fat and mineral additions to a corn-meal-oil-meal mixture did not increase the percentage of nitrogen retained as compared with the same grain mixture fed without such additions.

There was not always close agreement among individuals in their rates of growth and consequently in the production value of the protein mixtures tested. However, enough experiments were run with a given mixture to give confidence in the data, and from all the data the three highest results were selected for the final record. In all these experiments the plane of protein intake was kept fairly constant and between 12 to 15 per cent. In one instance it was 11.8 per cent of the ration (experiments on Pigs 13 to 15), and in two other cases slightly above 17 (experiments on Pigs 40 to 45). On these planes of protein intake, 12 to 15 per cent, the amount of nitrogen absorbed and retained by a 50 lb. pig was below the maximum possible. This was done purposely, as in no other way could an accurate efficiency figure be obtained. If more nitrogen is ingested than is necessary to meet the maintenance requirements and the maximum protein constructive powers of the animal the excess of nitrogen will appear in the excreta and necessarily lower the percentage of efficiency.

On a corn and milk ration and a 13 per cent protein level (16.4 gm. of nitrogen daily) a 50 lb. pig will retain approximately 10 gm. of nitrogen per day or 60 per cent of the total nitrogen ingested. Even this figure may not be the maximum retention rate, but such a high value was never reached with any other protein mixture tried except with another grain and milk. For this reason we feel certain that the production values below 60 to 65 per cent which were obtained represent the maximum efficiency for maintenance and growth of the particular protein mixture used.

Special attention should be called to the influence in the rations of a roughage such as alfalfa on the production value of a protein mixture. A roughage, necessarily high in fiber, will produce large fecal residues. With a copious absorption by this fibrous residue of increased intestinal secretions, the amount of nitrogen eliminated by way of the gut and consequently lost to the animal is greatly increased. Compare the fecal nitrogen lost in experiments on Pigs 7 to 9, on a corn-milk-alfalfa ration, and experiments on Pigs 25 to 26, on a corn-milk ration. The fecal nitrogen eliminated in the former series was two and a half times as large as in the corn-milk series. Another factor involved in the above consideration is the lower solubility in the intestinal juices of the nitrogen of alfalfa as compared with the nitrogen of corn-meal or milk. With 25 per cent of the dry matter of the ration coming from alfalfa the production value was lowered 20 per cent. A fact of this kind must be taken into consideration in planning rations of high protein efficiency. Where it is extremely desirable and in fact positively necessary to provide roughage in the ration of most animals for long continued physiological well being, the maximum rates of protein construction must be met by the use of higher planes of protein intake. While corn-meal and milk form an excellent protein mixture of high production value, due in part to low fecal residues, yet such a diet, without roughage, would terminate the life of a growing pig in 5 months.

The production values of the *absorbed* nitrogen of the two rations, corn-milk and corn-milk-alfalfa, are not so different. One might be inclined to use this figure as the numerical expression of the production value of the protein mixture ingested. This, we believe, would be a true expression of the nutritive worth of the

proteins, but such figures would be of less direct or practical value. The proteins are always fed in company with the other constituents of the natural material, and if these modify the amount of total nitrogen left to the organism, as is the case where variable amounts of fiber are ingested, then it would seem to us that this fact must be considered in arriving at the production value of any protein mixture as it occurs in natural feeds; consequently in Table I the production value of the protein mixtures is given in terms of total ingested nitrogen, as well as total absorbed nitrogen.

The net energy requirements of a 50 lb. pig for both maintenance and growth as given by Armsby[4] is 1.38 therms. No data are available on the net energy content of a number of the feeds used in our work. For example, the production value of alfalfa per 100 lbs. for a ruminant is given as 34 therms, for a horse 48 therms, but no figure is given for swine. In our calculations the figure used for alfalfa was that given for the horse and undoubtedly is somewhat too low. In the absence of available data on the energy value for swine of the other feeds used in our work recourse was made to the figures available for the ruminant or horse. Since the net energy of a feed for a ruminant or horse is somewhat lower than that for swine, due to increased losses incident to intestinal fermentation, the calculated net energy of our rations may have been somewhat lower than what it really was. Probably in every case the energy requirement of the experimental animals was fully met. Further, we were guided somewhat by the appetites of our animals and in consequence of this the ration actually consumed invariably provided more net energy than is called for in Armsby's standard for growing swine. In Table I the column of therms provided represents net energy values.

DISCUSSION.

The data disclose some facts of considerable practical importance. Cereal grain proteins of low production value are not appreciably increased in efficiency by supplementing with a corn kernel protein concentrate, such as gluten feed, or by a legume roughage, such as alfalfa; even the proteins of flaxseed meal (oil-

[4] Armsby, H. P., The nutrition of farm animals, New York, 1917, 713.

meal) when constituting 50 per cent of the ration did not increase the efficiency of those of the corn kernel. But when the flaxseed meal proteins constituted 20 to 25 per cent of the ration and the other 75 to 80 per cent came from the corn kernel there was an increased efficiency. The full data on oil-meal (flaxseed) proteins as supplements will be incorporated in a later paper.

The effective supplementing of the cereal proteins, cereal proteins plus alfalfa proteins, or cereal proteins plus cabbage and potato proteins was accomplished by additions of either milk or whey, or meat or fish proteins. The most effective supplementing was with milk and whey proteins (see charts). With but 16 per cent of the nitrogen coming from the whey proteins the efficiency of a corn-alfalfa-whey protein mixture was made nearly equal to that of a similar mixture in which 27 per cent of the nitrogen came from skim milk proteins in place of the whey proteins. The tankage proteins were not quite so effective in their supplementary relations as were those of milk or whey, although the inferiority was slight. Further, it was found that where 18 per cent of the nitrogen of a corn-alfalfa-tankage ration came from tankage the mixture was practically as efficient as where 35 per cent of the nitrogen came from tankage. It is to be expected that variations in the efficiency of the proteins of such commercial products as tankage, fish meal, meat scraps, etc., will occur, dependent upon what proportion of their total proteins is derived from the less active and incomplete albuminoid group of proteins, such as elastin, collagen, keratin, etc.

Meat crisps or fish meal fed with starch and serving as the sole source of nitrogen (experiments on Pigs 34 to 39) showed efficiencies of but 40 per cent, while milk proteins used under similar conditions showed a value of 60.

The protein mixture from corn and milk, or oats and milk showed production values of 60 to 65 per cent, with approximately 25 per cent of the nitrogen derived from skim milk, while the efficiency of a corn and tankage protein mixture, or a barley-tankage protein mixture was about 40 per cent. In the latter case approximately 30 per cent of the nitrogen was derived from tankage. It appears fairly certain that either milk or meat will supplement the proteins of the various cereal grains to approximately the same degree; that is to say, tankage when used to

supplement barley, oats, or corn will yield a protein mixture of similar production value. Likewise milk will supplement any of the cereal grains to a degree of approximately equal efficiency, but to a degree of greater efficiency than will tankage. This being true, then any differences observed in the rates of growth of animals receiving such mixtures will rest upon other deficiencies or limitations in ration or animal. This principle, once firmly established, leaves the choice of the cereal grain to be used in feeding for growth a matter only of cost.

The experiments on Pigs 46 to 54 touch human as well as animal nutrition. A protein mixture drawn from five common sources, namely rice, wheat, corn, potatoes,[5] and cabbage,[6] showed a low value for growth when the protein level was brought to a basis comparable to our other experiments, namely 13.3 per cent, by the use of a protein concentrate such as wheat gluten. The production value of this mixture was 19.4 per cent. When the nitrogen of the wheat gluten was displaced by an equal amount of meat crisps nitrogen the efficiency rose to 32.7, and when it was replaced by an equivalent in milk nitrogen it rose to 47 per cent. The efficiency of the latter mixture was not so high as a corn-grain-milk protein mixture alone, due largely to the greater fecal losses involved in the mixed ration containing cabbage.

[5] Potatoes were sliced and heated in the autoclave in steam at 15 pounds pressure for 20 minutes.

[6] The cabbage was dried, but uncooked.

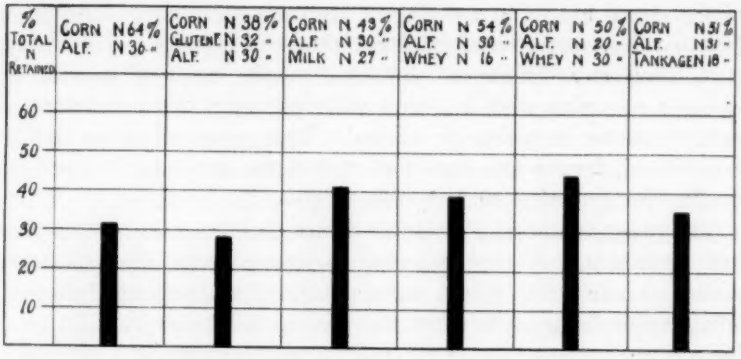

CHART 1. Maintenance and production values of some protein mixtures. Fed at approximately the same level, namely 12 to 15 per cent of the ration.

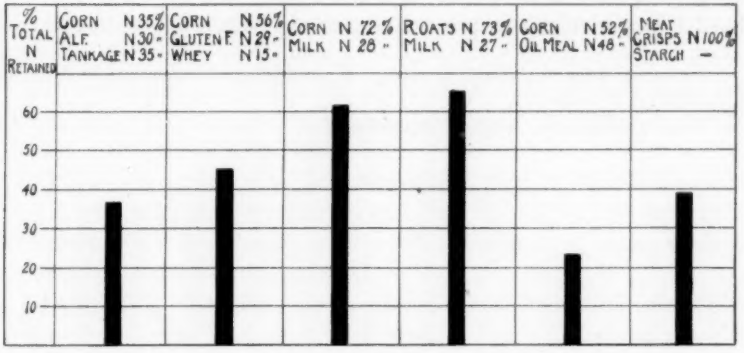

CHART 2. Maintenance and production values of some protein mixtures. Fed at approximately the same level, namely 12 to 15 per cent of the ration.

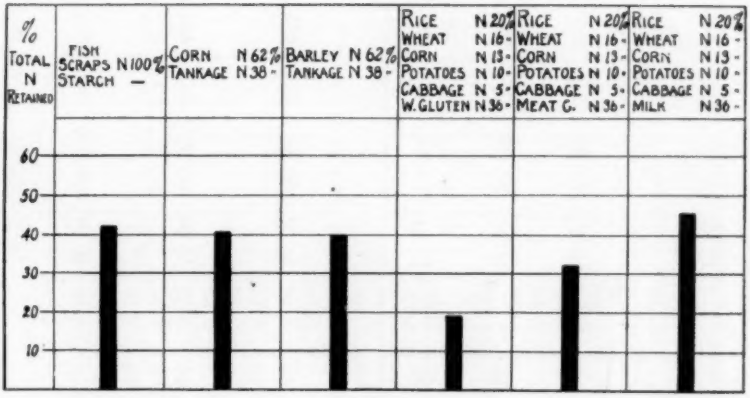

CHART 3. Maintenance and production values of some protein mixtures. Fed at approximately the same level, namely 12 to 15 per cent of the ration.

TABLE I.

Complete Data on Nitrogen Balance.

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily.		Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained.	Absorbed N retained.
	lbs.	*gm.*		*gm.*		*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	54	450 corn. 150 alfalfa.	1.36	7.65 4.26	11.91	74.43	12.4	285.8	85.5	101.6	34.6	49.3
2	56	450 corn. 150 alfalfa.	1.36	7.65 4.26	11.91	74.43	12.4	285.8	90.0	115.0	28.3	40.7
3	53	450 corn. 150 alfalfa.	1.36	7.65 4.26	11.91	74.43	12.4	285.8	105.9	87.6	32.3	51.8
Average											**31.7**	
4	60	385 corn. 140 gluten feed. 175 alfalfa.	1.54	6.54 5.40 4.97	16.91	105.68	15.1	405.8	127.2	161.0	28.9	42.2
5	65	385 corn. 140 gluten feed. 175 alfalfa.	1.54	6.54 5.40 4.97	16.91	105.68	15.1	405.8	171.0	122.7	27.6	47.7
6	61	385 corn. 140 gluten feed. 175 alfalfa	1.54	6.54 5.40 4.97	16.91	105.68	15.1	405.8	144.0	139.7	27.6	42.8
Average											**28.0**	

7	60	420 corn. 175 alfalfa. 900 cc. skim milk, 10 per cent solids.	1.50	7.14 4.97 4.50	16.61	103.81	15.1	398.6	123.0	101.1	43.7	63.3
8	62	420 corn. 175 alfalfa. 900 cc. skim milk, 10 per cent solids.	1.50	7.14 4.97 4.50	16.61	103.81	15.1	398.6	142.0	97.1	40.0	62.1
9	58	420 corn. 175 alfalfa. 900 cc. skim milk, 10 per cent solids.	1.50	7.14 4.97 4.50	16.61	103.81	15.1	398.6	129.0	106.4	40.9	60.5
Average											41.5	
10	60	525 corn. 175 alfalfa. 2,000 cc. whey, 6.6 per cent solids.	1.58	8.92 4.97 2.40	16.29	101.81	12.2	390.9	143.5	84.0	41.8	66.0
11	55	525 corn. 175 alfalfa. 2,000 cc. whey, 6.6 per cent solids.	1.58	8.92 4.97 2.40	16.29	101.81	12.2	390.9	157.0	86.4	37.7	63.0
12	66	525 corn. 175 alfalfa. 2,000 cc. whey, 6.6 per cent solids.	1.58	8.92 4.97 2.40	16.29	101.81	12.2	390.9	149.0	94.5	37.7	60.9
Average											39.1	

TABLE I—*Continued.*

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily.		Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained.	Absorbed N retained.
	lbs.	*gm.*		*gm.*		*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
13	53	500 corn. 125 alfalfa. 4,000 cc. whey, 6.6 per cent solids.	1.90	8.50 3.55 4.80	16.85	105.31	11.8	404.4	99.9	112.7	47.4	62.9
14	55	500 corn. 125 alfalfa. 4,000 cc. whey, 6.6 per cent solids.	1.90	8.50 3.55 4.80	16.85	105.31	11.8	404.4	121.4	98.1	45.7	65.3
15	51	500 corn. 125 alfalfa. 4,000 cc. whey, 6.6 per cent solids.	1.90	8.50 3.55 4.80	16.85	105.31	11.8	404.4	124.0	115.1	40.8	58.9
Average											44.6	
16	62	497 corn. 175 alfalfa. 28 tankage.	1.38	8.45 4.97 2.94	16.36	102.25	14.6	392.6	160.0	99.1	34.0	57.3
17	72	568 corn. 200 alfalfa. 32 tankage.	1.38	9.65 5.68 3.36	18.69	116.81	14.6	448.5	170.0	120.4	35.2	56.7
18	58	568 corn. 200 alfalfa. 32 tankage.	1.38	9.65 5.68 3.36	18.69	116.81	14.6	448.5	172.4	105.2	38.1	61.5
Average											35.8	

Average											35.8	
19	53	350 corn. 175 alfalfa. 56 tankage. 119 starch.	1.48	5.95 4.97 5.88	16.80	105.0	15.0	403.2	145.6	110.5	36.4	57.1
20	60	350 corn 175 alfalfa. 56 tankage. 119 starch.	1.48	5.95 4.97 5.88	16.80	105.0	15.0	403.2	152.0	97.9	38.0	61.0
21	46	350 corn. 175 alfalfa. 56 tankage. 119 starch.	1.48	5.95 4.97 5.88	16.80	105.0	15.0	403.2	160.1	87.1	38.6	64.0
Average											37.7	
22	70	550 corn. 125 gluten feed. 2,000 cc. whey, 6.6 per cent solids.	2.10	9.35 4.82 2.40	16.57	103.5	12.8	397.6	44.6	166.3	46.9	52.9
23	73	550 corn. 125 gluten feed. 2,000 cc. whey, 6.6 per cent solids.	2.10	9.35 4.82 2.40	16.57	103.5	12.8	397.6	66.8	154.3	44.3	53.3
24	70	550 corn. 125 gluten feed. 2,000 cc. whey, 6.6 per cent solids.	2.10	9.35 4.82 2.40	16.57	103.5	12.8	397.6	59.4	163.0	44.0	51.8
Average											45.1	

TABLE 1—*Continued.*

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily.		Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained.	Absorbed N retained.
	lbs.	*gm.*		*gm.*		*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
25	60	700 corn. 900 cc. milk, 10 per cent solids.	2.09	11.90 4.50	16.4	102.5	13.0	393.6	52.6	89.0	63.9	73.8
26	63	700 corn. 900 cc. milk, 10 per cent solids.	2.09	11.90 4.50	16.4	102.5	13.0	393.6	50.0	107.4	60.0	68.7
27	70	700 corn. 900 cc. milk, 10 per cent solids.	2.09	11.90 4.50	16.4	102.5	13.0	393.6	57.7	94.6	61.3	71.8
Average											**61.7**	
28	97	750 rolled oats. 300 starch. 1,350 cc. skim milk.	3.12	18.78 6.75	25.53	159.6	13.4	612.7	61.0	148.4	65.8	**73.1**
29	95	750 rolled oats. 300 starch. 1,350 cc. skim milk.	3.12	18.78 6.75	25.53	159.6	13.4	612.7	41.3	156.2	67.7	72.6
Average											**66.7**	

30	49	480 corn. 120 oil-meal.	1.69	6.24 5.88	12.12	75.75	12.6	292.8	94.0	125.9	24.9	36.6
31	48	480 corn. 120 oil-meal.	1.69	6.24 5.88	12.12	75.75	12.6	292.8	79.9	140.8	24.6	33.8
Average											24.8	
32	40	400 corn. 100 oil-meal. 25 butter fat. 8 Ca acetate. 5 NaCl.	1.69	5.20 4.90	10.10	63.10	12.6	242.4	57.2	125.6	24.5	32.1
33	45	400 corn. 100 oil-meal. 25 butter fat. 8 Ca acetate. 5 NaCl.	1.69	5.20 4.90	10.10	63.10	12.6	242.4	64.8	119.7	23.8	32.6
Average											24.6	
34	42	135 meat crisps. 565 corn starch.	1.78	15.6		97.5	13.9	374.4	14.5	215.8	38.4	40.0
35	43	140 meat crisps. 560 corn starch.	1.78	16.2		101.2	13.0	388.8	20.0	210.3	40.7	42.9
36	56	140 meat crisps. 560 corn starch.	1.78	16.2		101.2	13.0	388.8	21.1	214.9	39.3	41.5
Average											39.5	

TABLE I—*Continued.*

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily.	Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained.	Absorbed N retained.
	lbs.	*gm.*		*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
37	55	200 fish scraps. 500 corn starch.	1.77	16.6	103.7	14.8	398.4	50.2	179.4	42.3	48.4
38	60	200 fish scraps. 500 corn starch.	1.77	16.6	103.7	14.8	398.4	48.3	180.0	42.7	48.5
39	42	176 fish scraps. 424 corn starch.	1.53	14.6	91.2	15.2	350.4	76.4	131.9	40.5	51.8
Average										**41.8**	
40	53	535 corn. 65 tankage.	1.55	10.4 6.3 } 16.7	104.3	17.3	400.8	121.7	117.8	40.2	57.7
41	63	535 corn. 65 tankage.	1.55	10.4 6.3 } 16.7	104.3	17.3	400.8	128.1	122.4	37.5	55.1
42	56	535 corn. 65 tankage.	1.55	10.4 6.3 } 16.7	104.3	17.3	400.8	100.5	128.1	43.2	57.4
Average										**40.3**	
43	57	535 barley. 65 tankage.	1.50	10.5 6.3 } 16.8	105.0	17.5	403.2	133.5	108.4	40.0	59.7
44	47	535 barley. 65 tankage.	1.50	10.5 6.3 } 16.8	105.0	17.5	403.2	139.5	106.9	38.8	59.4
45	57	535 barley. 65 tankage.	1.50	10.5 6.3 } 16.8	105.0	17.5	403.2	135.8	104.2	40.4	61.0
Average										39.7	

46	45	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 38.4 wheat gluten.	1.44	2.69 2.22 1.77 1.30 0.70 4.64	13.32	83.2	15.4	319.6	71.8	177.2	22.0	28.4
47	45	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 38.4 wheat gluten.	1.44	2.69 2.22 1.77 1.30 0.70 4.64	13.32	83.2	15.4	319.6	86.5	176.8	14.4	24.1
48	45	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 38.4 wheat gluten.	1.44	2.69 2.22 1.77 1.30 0.70 4.64	13.32	83.2	15.4	319.6	69.1	180.7	21.8	27.8
Average											19.4	

TABLE I—*Concluded.*

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily.		Total protein fed daily.	Protein in ration.	Total N fed (24 days).	N in feces (24 days).	N in urine (24 days).	Total N retained	Absorbed N retained.
	lbs.	*gm.*		*gm.*		*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
49	42	136.8 rice.		2.69								
		136.8 wheat.		2.22								
		136.8 corn.		1.77								
		60 potato.	1.44	1.30	12.98	81.2	15.0	312.2	70.8	132.9	34.7	46.0
		30 cabbage.		0.70								
		38.4 meat crisps.		4.30								
50	44	136.8 rice.		2.69								
		136.8 wheat.		2.22								
		136.8 corn.		1.77								
		60 potato.	1.44	1.30	12.98	81.2	15.0	312.2	61.2	148.3	32.8	40.9
		30 cabbage.		0.70								
		38.4 meat crisps.		4.30								
51	44	136.8 rice.		2.69								
		136.8 wheat.		2.22								
		136.8 corn.		1.77								
		60 potato.	1.44	1.30	12.98	81.2	15.0	312.2	66.7	150.1	30.5	38.8
		30 cabbage.		0.70								
		38.4 meat crisps.		4.30								
Average											32.7	

52	38	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 920 cc. milk.	1.44	2.69 2.22 1.77 1.30 0.70 4.60	13.28	83.0	14.0	317.7	66.6	105.3	45.9	58.0
53	39	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 920 cc. milk.	1.44	2.69 2.22 1.77 1.30 0.70 4.60	13.28	83.0	14.0	317.7	53.3	107.6	49.3	59.3
54	39	136.8 rice. 136.8 wheat. 136.8 corn. 60 potato. 30 cabbage. 920 cc. milk.	1.44	2.69 2.22 1.77 1.30 0.70 4.60	13.28	83.0	14.0	317.7	53.3	118.8	45.8	55.0
Average											47.0	

A REVISION OF THE COPPER PHOSPHATE METHOD FOR THE TITRATION OF SUGAR.

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While preparing for this season's class work in sugar titrations according to the method of Folin and McEllroy[1] it was found that the salt mixtures prepared from the same lot of chemicals by different individuals did not give identical results. Many of these salt mixtures behaved very much like those recently obtained by Haskins[2] when he used sodium thiocyanate instead of potassium thiocyanate. When the theoretical quantity of sugar (25 mg. of dextrose) was added the copper was completely reduced in 3 minutes or even less whereas complete reduction should not occur in less than about 4 minutes. The results obtained were the same whether the thiocyanate used in preparing the reagent was the sodium or the potassium salt; but, in the interest of economy, the amount of sodium thiocyanate employed can readily be reduced by 15 to 20 per cent, without encountering red cuprous oxide and without altering the titration value. The variations encountered in the sugar titrations were due partly to the use of granulated disodium phosphate which had lost considerable water of crystallization. The most important cause of the divergent results obtained was due, however, to variations introduced by different individuals in the manner of preparing the salt mixtures. In order to obtain concordant results it is almost indispensable to first mix the phosphate and thiocyanate so as to permit the thiocyanate to abstract enough water from the phosphate to give a solution of the thiocyanate before mixing in the sodium carbonate. The solid salt mixture for the sugar titration of Folin and McEllroy should therefore be made as follows.

[1] Folin, O., and McEllroy, W. S., *J. Biol. Chem.*, 1918, xxxiii, 513.

[2] Haskins, H. D., *J. Biol. Chem.*, 1919, xxxvii, 303.

Powder in a large mortar 200 gm. of crystallized disodium phosphate ($HNa_2PO_4.12\ H_2O$) and sprinkle over it about 50 gm. of sodium thiocyanate (or 60 gm. of potassium thiocyanate). Mix for 10 minutes with mortar and spoon, giving a uniform semi-liquid paste. Add about 120 gm. of monohydrated sodium carbonate (or 100 to 110 gm. of anhydrous carbonate) and mix with mortar and spoon until a rather fluffy granular powder is obtained. Add 5 gm. of the salt mixture to 5 cc. of the copper sulfate solution; if any black specks are formed, even temporarily, the mixing is incomplete; a certain amount of green color is, however, practically unavoidable when this test is applied. If no black coloration is obtained the salt will give substantially correct titration values and can be bottled at once, though we usually leave it in the mortar for a few hours or over night (covered with paper) and then mix once more before transferring to bottles. In stoppered bottles the mixture keeps indefinitely.

The sugar titration according to the directions of Folin and McEllroy could however justly be criticised on the ground that the green color obtained when the salt mixture is added to the 6 per cent copper sulfate solution does undoubtedly represent reduction of copper sulfate by the thiocyanate. Correct results are obtained only because the copper sulfate solution has been made strong enough to compensate for the loss due to the thiocyanate reduction. Analogous criticisms can be made of every sugar titration by alkaline copper solutions for in all of these variable amounts of sugar are destroyed by the alkali and the loss of some copper is no more serious than the loss of reducing action on the part of the sugar. In the sugar titration of Folin and McEllroy the loss of active copper is visible; it is also both measurable and preventable and the main object of this communication is to describe a modification of the method by which the unimportant but disconcerting loss of copper is eliminated.

The reduction of copper sulfate by the thiocyanate does not occur in alkaline solutions. The thiocyanate is so rapidly soluble in water (or copper sulfate solution) that a certain amount of reduction has taken place before the carbonate-phosphate mixture has had time to make the copper sulfate solution alkaline. The loss of copper thus occurring amounts to about 1.5 per cent. By first adding a little sodium carbonate alone to the copper sul-

fate solution (1 cc. of saturated sodium carbonate) the copper is precipitated and the solution is rendered alkaline and the reaction between the copper sulfate and the thiocyanate is eliminated. The proposed modification requires, of course, that the amount of copper sulfate used for each titration be somewhat diminished; the 6 per cent solution must be reduced to one containing 5.9 per cent of copper sulfate. The reagents required for the revised sugar titration are:

1. The salt mixture prepared as described above, containing 200 gm. of crystallized disodium phosphate, 50 gm. of sodium thiocyanate, and 100 gm. of anhydrous sodium carbonate, or 120 gm. of monohydrated sodium carbonate. For use with the revised titration it is not important to keep the mixture damp and the reagent is more easily weighed or measured if allowed to "weather" until so dry it can be easily poured from one test-tube to another.

2. Approximately saturated sodium carbonate solution (14 to 20 per cent Na_2CO_3).

3. A copper sulfate solution containing 59 gm. of $CuSO_4.5H_2O$ and 2 cc. of concentrated sulfuric acid per liter.

Complete reduction of 5 cc. of this copper solution in 4 to 6 minutes is obtained with 25 mg. of dextrose.

Concerning the titration (which is best made in large, hard glass test-tubes) we have little to add to the description given in the original method. It is advantageous to use test-tubes which are dry, or which have been rinsed with alcohol, because with such test-tubes the bumping is much diminished. We now use exclusively special 5 cc. sugar burettes graduated in 0.02 cc. together with accessory tips which are very fine. The burettes are filled by suction.[3]

Transfer 5 cc. of the 5.9 per cent copper sulfate solution to a test-tube. Add first approximately 1 cc. of saturated sodium carbonate solution. Shake for a moment and then add 4 to 5 gm. of the phosphate-carbonate-thiocyanate mixture. Heat gently with shaking until all the salts have dissolved except for a few isolated particles of sodium carbonate. A clear solution is usually obtained in less than one minute at temperatures which need not exceed 60°C. Add sugar solution or undiluted urine (0.4 cc.

[3] These burettes, with accessories, are now made by the Emil Greiner Company, New York.

to 1.0 cc.), heat fairly rapidly to boiling, and then boil *very gently* so as not to drive off too much water. When the mixture begins to bump add a pebble. The following observations, though subjected to some variations, may prove helpful for the avoidance of needless repetitions in connection with the titration.

When 25 mg. of dextrose are added at once, 90 to 93 per cent of the copper will be reduced in 2 minutes and about 98 per cent in 3 minutes. When only 1 per cent of unreduced copper is left, the solution is still so blue that it is impossible to overlook it. This point is most easily demonstrated by adding 1 cc. of carbonate solution and 5 gm. of the solid reagent to 5 cc. of a copper sulfate solution representing a hundredfold dilution of the original copper sulfate solution. With the full required amount of sugar present at the beginning, the boiling solution becomes suddenly turbid within 5 seconds after the boiling point has been reached. If the boiling contents of the test-tube do not thus suddenly become filled with the cuprous thiocyanate precipitate within the first 15 seconds of boiling then less than half of the required sugar has been added and more of the sugar solution or urine should be added without further delay. On the other hand, when an excess of sugar has been inadvertently added at the beginning of the process it is advisable to note the time required for complete decolorization of the copper, for this time (see table) can serve as a guide to the quantity of solution to be introduced at the beginning of the next titration.

TABLE I.

Time of Boiling Required for Complete Reduction of Copper Solution by an Excess of Dextrose.

Dextrose.	Boiling time.	
mg.	*min.*	*sec.*
50	0	25
40	0	40
35	0	55
30	1	20 to 30
27.5	1	30 to 55

The only time restriction called for in connection with the final titration is that complete reduction must not occur in less

than 4 minutes boiling; in other words, it makes practically no difference in the result (at the most, 1 per cent) if the boiling period be prolonged to 8 or 9 minutes, provided that the boiling be gentle enough to prevent excessive concentration. The volume of the solution in the test-tube should not become less than 6 to 7 cc.

The modification of the titration of dextrose described above is also applicable to the determination of the lactose in milk, as well as for the titration of other sugars.

THE SCURVY OF GUINEA PIGS.*

III. THE EFFECT OF AGE, HEAT, AND REACTION ON ANTISCORBUTIC FOODS.

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Recently considerable experimental work has been done on the effect of heat on vitamines, more particularly on the water-soluble vitamine. In a recent article Denton and Kohman (1) come to the conclusion that "ordinary methods of cooking do not perceptibly injure the nutritive value of carrots." These experiments were carried out on rats, which preclude a satisfactory consideration of experimental scurvy.

In the following experiments guinea pigs weighing about 200 to 250 gm. were employed. As will be seen from Chart 1, it was found that in the course of cooking, carrots lose a very large part of their antiscorbutic potency; that whereas 35 gm. of carrots were able to protect a guinea pig from scurvy when added to its ration, the same amount of carrots which had been cooked for about 45 minutes had lost its potency in this respect. An attempt was made to prevent this loss by acidulating the water by the addition of vinegar. As will be noted from Chart 3, however, the animals came down with scurvy within the usual period.

Further experiments demonstrated the fact that from a nutritional standpoint carrots cannot be looked upon as a uniform article of diet; that there is a marked difference in various lots of carrots and probably also of other vegetables, according to whether they are fresh and young, or are old. It was found, for example, that if, instead of employing the carrots which were ordinarily fed

* A preliminary report of this work was published in the *Proc. Soc. Exp. Biol. and Med.*, 1919, xvi, 52.

to our laboratory animals, we gave the same amount of fresh young carrots, plucked only a few days previously and cooked, not only did the animals not develop scurvy, but that they gained steadily in weight for a long period (Chart 2). These carrots could be boiled only for about twenty minutes, as they began to disintegrate if cooked longer; they were then allowed to stand in the boiled water for about one-half hour. Old carrots have therefore a twofold disadvantage in that they contain less antiscorbutic vitamine than the young vegetable, and that they require additional boiling, which still further decreases their limited supply of this essential food factor.

The freshness and age of the vegetables sufficed also to enable them to retain their antiscorbutic potency after dehydration. In previous articles (2, 3) it was stated to have been our experience not only in animal scurvy, but in human scurvy as well, that dehydrated vegetables as purchased in the open market or prepared in the household were without noticeable value in the cure of scurvy. Under favorable conditions certain vegetables can withstand dehydration. This has been conclusively shown by Holst (4) and by Cohen and Mendel (5) in connection with cabbage, and in relation to tomatoes by Givens and McClugage (6). After noting the marked difference between old and young carrots in their ability to withstand the cooking process, we decided to make a similar test in regard to drying, and accordingly had some carrots dehydrated the day they were plucked.[1] It was found that a *per capita* daily allowance of 4.5 gm. of the dried carrots added to the ration was sufficient not only to protect pigs against scurvy but to cure them of this disorder (Chart 3). This amount is equivalent to 35 gm. of fresh carrots, the same quantity which failed as an antiscorbutic when the commercially dehydrated carrots were employed. The dehydrated carrots were kept at room temperature in sealed cartons for a month before the experiment was begun, and had been dried for about 3 months at the end of the test.

This factor of the freshness and age of vegetables or fruit has not been considered in relation to their content of the antiscor-

[1] These vegetables were furnished and prepared by Mr. C. Eckroth. The dehydration was carried out by the Mrs. Oliver Harriman Food Research Laboratories, the same plant which dehydrated the vegetables referred to in the previous papers.

butic or other essential food factors. From the above experiments it is evident that it plays an important rôle, and that in the course of aging at least the antiscorbutic vitamine is greatly lessened. It will have to be given consideration in judging the results in experimental scurvy. This variable factor renders it very difficult to prepare a table of the comparative antiscorbutic value of various foods. Where this is attempted, as recently by Chick and Hume (7), all the vegetables and fruits should be uniform in their development and freshness. It is evident that it becomes a matter of prime importance for the dehydrating industry to make use of such vegetables. It may come to pass that by observing this precaution manufacturers will attain the goal of furnishing a product comparable to the raw food. It should be mentioned that in our experiments there was an additional factor to be considered; namely, that the vegetables were not only fresh but young, and therefore more cellular than the older vegetables.

Experiments were carried out to determine whether the water in which the carrots were cooked contained the antiscorbutic factor. It has been reported by Daniels and McClurg (8) that the liquor from cooked beans contains water-soluble vitamine. Our experiments showed that the water in which the carrots were cooked was of little or no value in protecting guinea pigs from scurvy. The water was given, as will be seen from Charts 4 and 5, in the amount of 40 cc. *per capita* daily, but did not delay the onset of the disease. This addition to the dietary seemed to prevent the marked loss of weight, probably due in part to its salt content. No appreciable difference was noted between the liquor in which old or young carrots had been cooked (Charts 4 and 5).

In the experiments carried out at the Lister Institute by Chick and Hume and other workers, dried milk has been made use of as part of the standard diet in bringing about scurvy in guinea pigs. This food was added to the dietary in order to render it complete and because it was found that this milk was devoid of antiscorbutic power. The inference should not be drawn from these experiments, however, that all dried milk is the same in this regard. It would be an error to infer that milk necessarily loses its antiscorbutic potency when it is reduced to a dry state. In mak-

ing an experimental test of this question we employed a brand of milk which had been dried at a temperature of 116°C. for a period of a few seconds. This milk powder was diluted with eight volumes of water and given to the equivalent of 80 cc. of fresh milk for each guinea pig. This amount was selected as it had been found in previous experiments that 80 cc. of fresh raw milk sufficed to protect a guinea pig from scurvy.

Numerous experiments of this kind were carried out. One of them is shown in Chart 5. It will be seen that five guinea pigs which developed scurvy on a diet of hay and oats and 40 cc. of carrot water were rapidly cured, and gained in weight when the dry milk was added to their dietary. This and similar experiments led to the conclusion that milk which has been dried in this manner (the Just-Hatmaker process) loses little of its antiscorbutic factor. This inference was confirmed by finding that infantile scurvy could be cured by giving dried milk of this variety (2).

In a preliminary communication we reported that canned tomatoes are an excellent antiscorbutic (9). This opinion has been borne out by tests on animals as well as clinical experience. In a recent publication on infantile scurvy it was shown that one ounce of canned tomatoes is sufficient to protect an infant from scurvy and that this food is an excellent antiscorbutic for use in infant feeding and can well replace orange juice, which is so much more expensive. We have carried out an extended series of experiments to ascertain the amount of strained canned tomatoes which it is necessary to add to our ration to protect the guinea pig. It will be seen (Chart 6) that when 1.5 cc. of this food is added to the dietary of each pig scurvy supervenes, but that when this amount is increased to 5 cc. daily, the animals recover and continue to gain in weight for a period of months. Further experiments showed that when only 3 cc. *per capita* are used some of the animals developed scurvy, whereas others escaped this disorder. If the tomato is boiled it loses somewhat in efficacy, although even under these conditions 5 cc. were found to be sufficient to protect the pigs (Chart 7). It may be added that the tomatoes which were made use of had been canned almost one year previously.

In Chart 7 graphs are reproduced which are composites of three series of experiments; one in which 10 cc. of canned tomato were added to the dietary, another in which 30 cc. were added, and a third in which 60 cc. were given. These experiments were prolonged for 4 to 6 months in order to ascertain whether any symptoms of scurvy would eventually become manifest. All animals, however, remained entirely free from this disorder and gained steadily in weight. The group which received only 10 cc. of tomato thrived just as well as that which received 60 cc., showing that the antiscorbutic quota was entirely satisfied by the smaller amount. It may be added, in this connection, that it was found by tests on pigeons that canned tomato contained considerable of the "antineuritic vitamine," and that pigeons suffering from polyneuritis could be cured by giving them 5 cc. daily of this foodstuff.

It has been stated by Harden and Zilva (10) that when orange juice is rendered slightly alkaline it loses its antiscorbutic potency. This proved to be the case under certain conditions. This question was investigated in relation to canned tomato and to orange juice, and it was found that shortly after having been rendered 0.05 N alkaline to phenolphthalein neither of these foods had lost an appreciable amount of its antiscorbutic power. 5 cc. of alkalinized tomato were still able to protect a guinea pig from scurvy (Chart 8), and 5 cc. *per capita* daily of freshly alkalinized orange juice were able to cure a group of pigs which had developed scurvy on another diet (Chart 9). This is in conformity with the efficacious results obtained in human scurvy by the intravenous injection of boiled orange juice which has been rendered faintly alkaline (11). If, however, 24 hours were allowed to elapse between the alkalinization and the feeding, instead of only $\frac{1}{2}$ to 1 hour, then a considerable amount of the antiscorbutic factor was lost. This is well illustrated in Chart 9, where, after the scurvy had been cured by means of freshly alkalinized orange juice, it redeveloped when we gave an equal amount of orange juice which had been alkalinized 24 hours previously. The same rule seems to hold for alkalinization as for heating; *i.e.*, the length of time the antiscorbutic food is subjected to the deleterious influence is fully as important as the intensity of the process.

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3. Hess, A. F., and Unger, L. J., *Am. J. Dis. Child.*, 1919, xvii, 221.
4. Holst, A., and Fröhlich, T., *Norsk. Mag. f. Laegevidensk*, 1916, viii, 989.
5. Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.
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9. Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1918, xvi, 1.
10. Harden, A., and Zilva, S. S., *Lancet*, 1918, ii, 320.
11. Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1918, xv, 141.

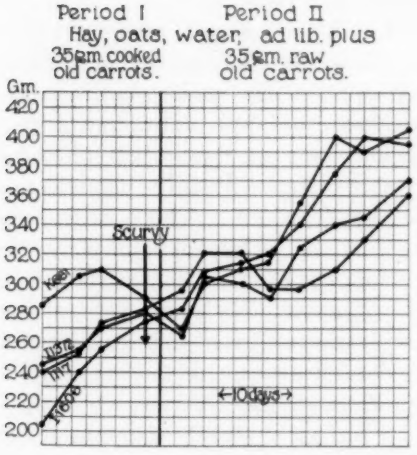

CHART 1. Showing the destruction of the antiscorbutic vitamine by cooking. The raw and the cooked carrots were of the same lot. The vegetables were cooked in an open vessel for about 45 minutes. (Scurvy developed in all 4 animals between the 14th and 16th days.)

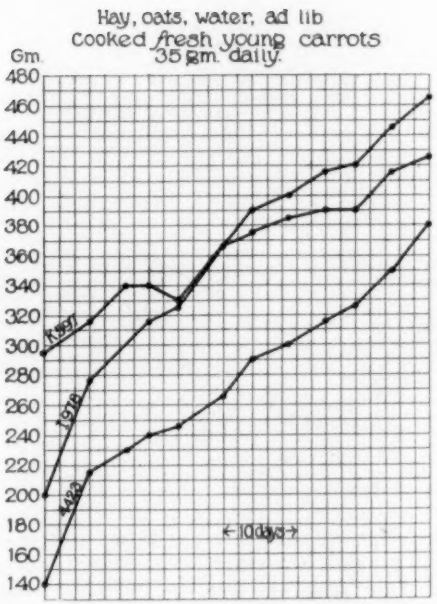

CHART 2. Guinea pigs were given the same amount *per capita* of cooked carrots as in the previous experiment (Chart 1), the only difference being that in the first test the carrots were old, whereas in the second they were young and fresh, having been plucked only a few days before being fed. No scurvy developed.

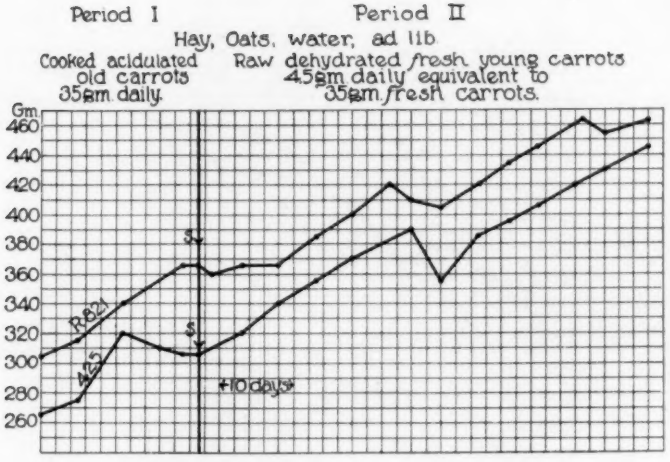

CHART 3. An attempt was made to retain the antiscorbutic vitamine by means of adding 10 per cent of vinegar to the water in which the old carrots were boiled. This failed, however, the animals developing scurvy.

The second or curative part of this experiment shows that if the carrots are young and fresh they will withstand dehydration. In a previous report it has been shown that this amount of commercially dehydrated carrots failed to protect guinea pigs. The carrots were dehydrated the same day they were plucked, at a temperature of about 71°C.

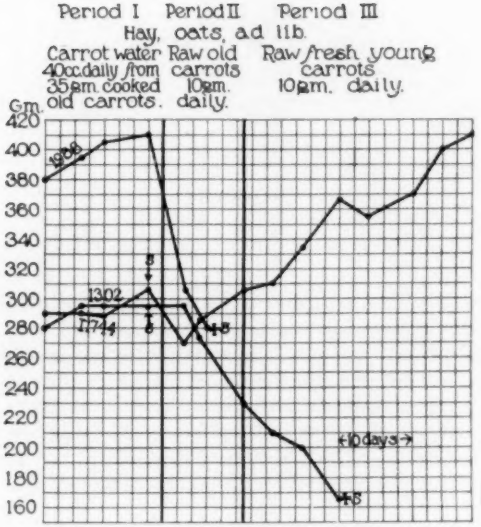

CHART 4. Period I of this experiment shows that the water in which the carrots have been cooked contains little if any antiscorbutic vitamine.

Period II demonstrates that 10 gm. daily of raw old carrots, such as are used ordinarily for feeding animals, supply an insufficient amount of this vitamine.

Period III shows that the same amount (10 gm.) daily of carrots which were young and freshly picked was able to cure scurvy.

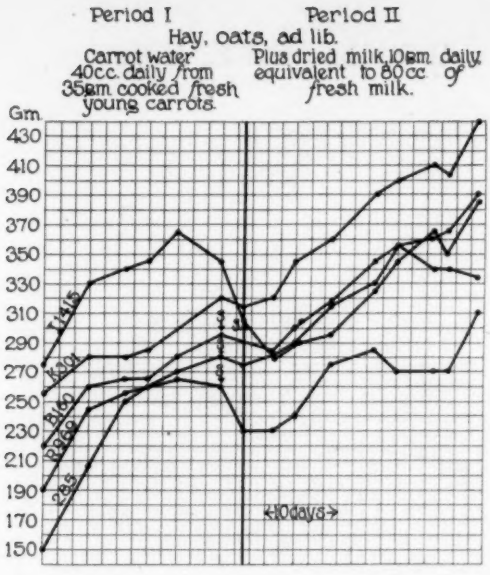

CHART 5. These guinea pigs developed scurvy in spite of receiving a large quantity of the water in which young carrots had been cooked for only 20 minutes. It will be noted, however, that although the pigs developed scurvy they did not lose weight, as is usually the case.

After they had developed definite scurvy they were given in addition the equivalent of 80 cc. of a dried milk prepared by being heated to about 116°C. for a few seconds. The addition of this milk to the diet cured the scurvy, showing that it had largely retained its antiscorbutic vitamine.

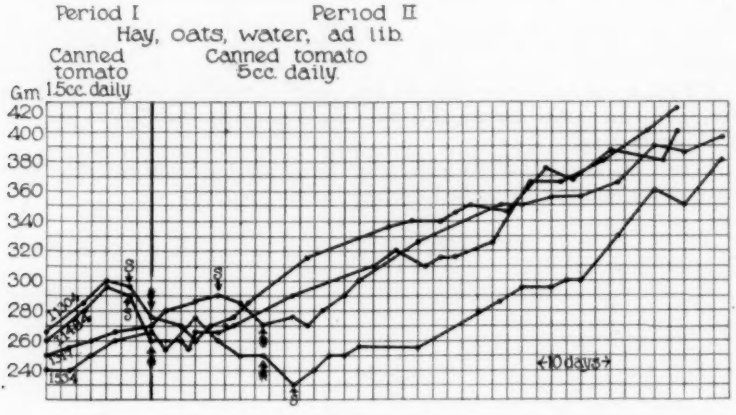

CHART 6. Guinea pigs were fed 1.5 cc. daily of canned tomato, in addition to their diet of hay, oats, and water. This was insufficient to protect them from scurvy. When the amount of tomato was increased to 5 cc. all symptoms of scurvy disappeared and they gained steadily in weight.

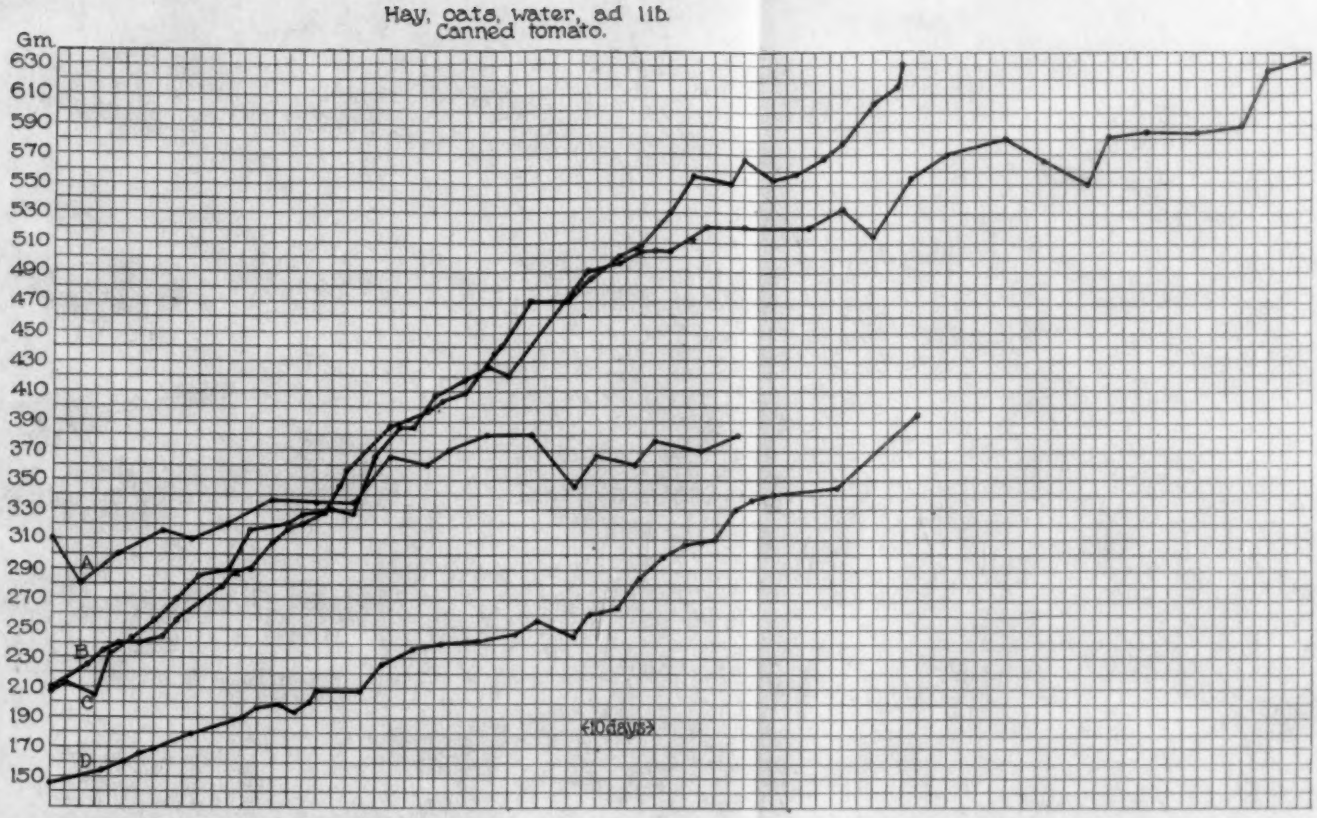

CHART 7. Each of these four curves is a composite representing an average of a group of guinea pigs, each containing 3 to 5 animals. Guinea pigs A received an addition to their diet of 5 cc. of canned tomato which had been boiled for 5 minutes. It will be seen that although scurvy did not develop, growth was not satisfactory.

Animals in groups B, C, and D received respectively additions of 60, 10, and 30 cc. of canned tomato to their diets. In all cases continuous growth was maintained for several months. It will be noted that the growth was about the same in the animals which received only 10 cc. as in those fed 60 cc. of tomato.

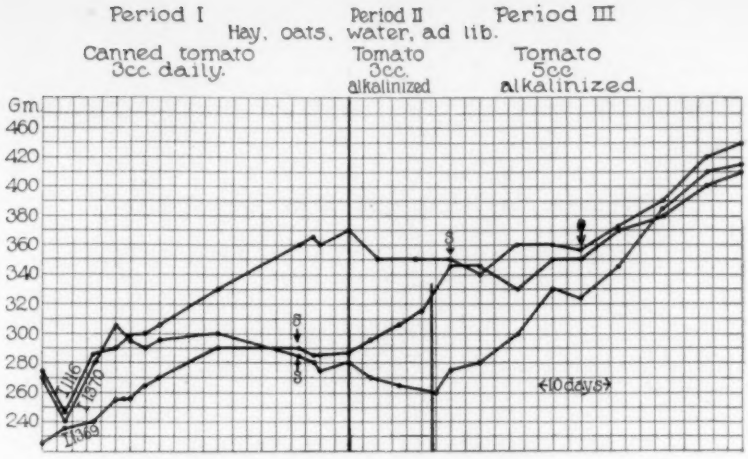

CHART 8. This chart shows that alkalinization does not destroy the antiscorbutic vitamine in canned tomato if it is fed soon after the sodium hydroxide is added. In two of the animals, indeed, it seemed to bring about temporary gain in weight. The tomato was rendered 0.05 N alkaline to phenolphthalein.

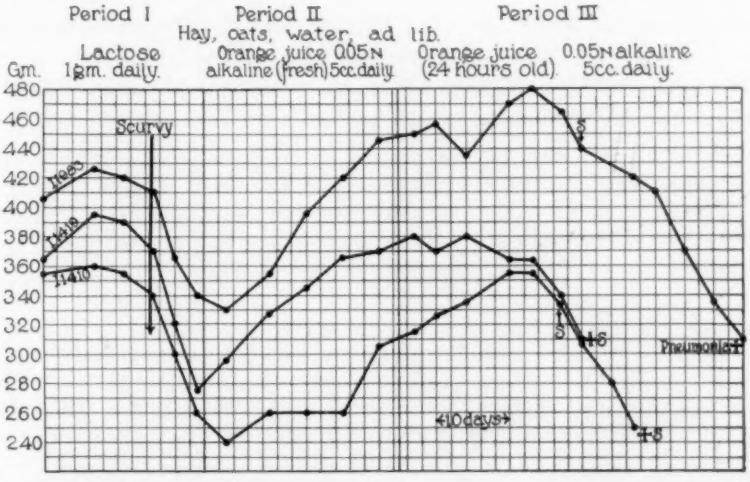

CHART 9. Animals which developed scurvy on a diet of hay, oats, and water plus lactose were cured by means of adding freshly alkalinized orange juice.

Period III of this experiment shows that when this alkalinized orange juice was allowed to stand in the refrigerator for 24 hours it lost its antiscorbutic potency and was unable to protect the guinea pigs from scurvy.

STUDIES OF EXPERIMENTAL SCURVY.

EFFECT OF HEAT ON THE ANTISCORBUTIC PROPERTIES OF SOME MILK PRODUCTS.*

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(Received for publication, April 24, 1919.)

The recent work of Chick and Hume,[1] Cohen and Mendel,[2] Hess and Unger,[3] and Givens and Cohen[4] gives full support to the earlier views of Holst and Frölich[5] that scurvy is the result of a deficiency of some nutritive factor in the diet. If we understand by the term vitamine the chemically unknown substances of food origin essential for the normal performance of the function of animal life there is perfect propriety in applying to the above mentioned factor the name antiscorbutic vitamine. As polyneuritis is caused by a lack of the water-soluble vitamine (antineuritic vitamine) and xerophthalmia by a lack of the fat-soluble vitamine (antixerophthalmic vitamine), scurvy is caused by a lack of the antiscorbutic vitamine.

Opposed to this view of the etiology of scurvy was the theory of McCollum and Pitz[6] which postulated that scurvy is related to

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1916–17, x, 141. Chick, H., Hume, M., and Skelton, R. F., *Lancet*, 1918, i, 1.

[2] Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

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[5] Holst, A., and Frölich, T., *J. Hyg.*, 1907, vii, 634; *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 1; *ibid.*, 1913, lxxv, 334.

[6] McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxii, 29.

intestinal putrefaction and retention of feces. As pointed out by Chick and Hume, the conclusions of the aforementioned investigators were based on data obtained from experiments where a vitiating factor was constantly operative, in that all the experimental animals were allowed milk *ad libitum*. As milk contains the antiscorbutic vitamine, the incidence of scurvy would consequently be dependent not only on the animal's idiosyncrasy in liking or disliking milk, but also on any change in experimental conditions which tended to modify the fluid intake. Chick and Hume were able to secure some protection against experimental scurvy in guinea pigs when 50 to 100 cc. of milk were given daily per individual, and when 100 to 150 cc. were given scurvy was entirely prevented. In all cases the milk allowance was superimposed on a basal ration of oats and bran.

This work on the rôle of milk we have repeated and confirmed, entirely preventing the development of scurvy with a diet of rolled oats, hay, and 100 cc. of whole milk per individual per day, and delaying the appearance of scurvy in guinea pigs for 18 weeks by a daily allowance per individual of 30 cc. of whole milk and a diet of rolled oats and dried hay. It is altogether probable, therefore, that the development of the theory of the intestinal origin of scurvy became connected with variations in the milk consumed by animals receiving different treatments.

The fact that Hess[7] was able to cure scurvy in guinea pigs by intravenous injection of orange juice gives incontrovertible evidence of the existence of an antiscorbutic vitamine. So conclusive has become the evidence for the existence in our foodstuffs of this third class of unknowns that we accept this point of view as now fully established.

The point of view that there does exist a relatively unstable antiscorbutic vitamine in our foods offers a satisfactory explanation of the prevalence of scurvy among infants fed milk of which the origin and heat treatment may have been variable. It opens for study the question of the variation in antiscorbutic vitamine content of milks produced under various conditions. The fact, first demonstrated by Holst and Frölich and further developed by Hess and by Givens and his associates, that mere

[7] Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 141.

drying of plant tissue destroys considerable quantities of this vitamine, possibly places the feeding of cows for special purposes, as for the production of a milk rich in the antiscorbutic factor, in a new light and calls for investigation. Further, with the recognition of a third class of vitamines, in addition to the fat-soluble (antixeropthalmic) and water-soluble (antineuritic) types, as essential to the life of certain species as man, monkey, and guinea pigs, comes the question of its relation to the life of other species of animals. Does the guinea pig need an abundance of this vitamine and therefore fail on a diet of corn-meal, peas, and dried alfalfa flour,—low in the antiscorbutic factor—while the rat, cow, or pig needs less or none of it, and therefore succeeds on such a ration? These are questions of great interest and fundamental to a complete understanding of all the factors involved in animal nutrition. A recent paper by Harden and Zilva[8] involves an investigation of this question with special reference to the rat.

The data presented in this paper involve a repetition of some of the earlier experiments conducted in this laboratory and, in addition, demonstrate that commercial condensed milks, commercial milk powders, and milks sterilized for 10 minutes at 120°C. have suffered the destruction of their antiscorbutic vitamine as determined experimentally with the guinea pig.

An excellent description of the symptomatology of guinea pig scurvy is given by Cohen and Mendel.[2] Our experience in following the course of this disease coincides very well with their observations. There occurs tenderness of the joints which provokes crying when touched; a progressive swelling of the affected joints, which, if not cured, become permanently hardened into exostoses; and a slowly increased inability to use the hind legs, which finally develops into an apparent paralysis of those parts. At this latter stage the animal lies on its side or back a considerable part of the time, taking the "face ache" position; the lower molars become loose, being easily picked out with a forceps, and in some cases there is an appearance of hemorrhage at the base of the lower incisors. At autopsy the junctions of the ribs and cartilages are enlarged, the bones are easily breakable, and conditions of hemorrhage or congestion are usually noticeable.

[8] Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 408.

The diet chosen as one invariably producing scurvy in the guinea pig was rolled oats and dried hay,—a mixture of June grass and timothy hay (see Chart 1). Fresh milk in the diet was avoided unless its consumption was quantitatively controlled because of its power, in an oat-hay diet, to prevent scurvy if consumed in large enough quantities. In the absence of hay and with a diet of rolled oats and fresh whole milk, involving an average daily consumption of 47 cc. of milk per individual, the guinea pig will develop scurvy (see Chart 2). In the presence of the hay a somewhat less amount of milk (30 cc.), consumed daily per individual, will prevent or at least greatly delay the onset of scurvy (see Chart 3). These results can be interpreted in two ways: either the hay still contains some of the antiscorbutic vitamine, which in addition to that in the milk and grain is sufficient to prevent the development of the disease, or the hay, as a source of roughage, or of mineral matter as pointed out by Pitz,[9] improves the conditions for normal nutrition to a degree sufficient for a smaller amount of the antiscorbutic factor to suffice. With a daily allowance of 30 cc. of fresh whole milk per individual, together with a ration of rolled oats and hay,—the latter always fed separately and *ad libitum*—there was a delayed development of scurvy, but absolute protection was not secured (see Chart 3). One animal, after giving birth to young, gradually developed the disease, while the other three animals showed symptoms of enlarged joints only after being on the ration 20 weeks.

Where 100 cc. of whole raw milk were allowed daily per individual and an average daily consumption per individual of 84 cc. was actually recorded with a basal ration of rolled oats and hay, absolute protection from scurvy was secured (see Chart 4). Even at the present writing and after a period of observation of 26 weeks, the animals appear in perfect condition. This work confirms the work of Chick and her associates on the quantitative relation of raw milk consumption to the development of a scorbutic condition in the guinea pig.

With either rolled oats and hay or rolled oats and milk we have been unable to prevent the development of scurvy by the administration of mineral oil. Lot 5, Chart 5, receiving a diet of

[9] Pitz, W., *J. Biol. Chem.*, 1918, xxxiii, 471.

rolled oats and hay was given 1 cc. of mineral oil per individual every other day after the first symptoms of scurvy appeared. They all died of the disease. Lot 6, Chart 6, on a diet of rolled oats and milk *ad libitum* (but an average daily consumption of 47 cc. per individual per day) developed scurvy from which they died at the end of 6 to 8 weeks. They had received 1 cc. of mineral oil per individual on alternate days starting from the time of the inauguration of the experiment. Even with a diet of rolled oats and hay, but with 1 cc. of mineral oil per individual administered daily from the beginning of the experiment, the development of scurvy could not be prevented (see Chart 7). There is but one conclusion possible from these data, namely, that mineral oil, acting as a laxative, cannot *per se* prevent the development of scurvy.

Phenolphthalein administration was also ineffective in preventing the development of scurvy on a diet of rolled oats and hay (see Chart 8). To this group 2 mg. of phenolphthalein per individual were administered on alternate days by gelatin capsule. Whether the administration of the phenolphthalein was begun at the initiation of the experiment (see Chart 9) or after the onset of scurvy symptoms, the final results were the same. All the animals died of scurvy. The more mature animals were as sensitive to the development of scurvy as those less mature and the disease terminated their lives in 5 weeks.

Pitz[9] had observed that lactose could prevent the development of scurvy in guinea pigs receiving a diet of rolled oats, a salt mixture, and fresh milk *ad libitum*. The fact that again milk was accessible to the animals for *ad libitum* consumption makes the conclusion questionable. The lactose may have increased the milk consumption or possibly may have carried some of the antiscorbutic vitamine, as the product was not especially purified in respect to its vitamine content. The results secured with Lot 10, Chart 10, where unheated lactose (Merck) was used with a diet of rolled oats and hay and with Lot 11, Chart 11, where the same quality and quantity of lactose were used, but after having been heated to 120°C. for 4 hours, are conclusive evidence that lactose in itself is not an antiscorbutic. All the animals in both groups died of scurvy in 4 to 5 weeks. They ate the rations well for 2 weeks as indicated by their increments in live weight.

Of special interest to us was the effect on the guinea pig of certain rations known by long experience to be satisfactory for cattle and swine. Rations consisting of intimate mixtures of rolled oats 84 parts, dried corn-stover 15 parts, and common salt 1 part (see Chart 12); or dried alfalfa hay 25 parts and rolled oats 75 parts (see Chart 13); or dried corn-stover 49 parts, rolled oats 50 parts, and common salt 1 part (see Chart 14); or corn-meal 35.7 parts, gluten feed 14.3 parts, dried corn-stover 49 parts, and common salt 1 part (see Chart 15); all produced scurvy in the guinea pig in from 4 to 5 weeks. Such rations gave no apparent nutritional disturbances over long continued use with cattle or swine. For example, we have reared cattle to maturity and obtained very successful reproduction on a ration of corn-meal 35.7 parts, gluten feed 14.3 parts, dried corn-stover 49 parts, and common salt 1 part. It is true, as far as we are aware, that scurvy has not been reported in cattle or swine, but it is difficult to conceive why any mammal should be exempt from the needs of this class of vitamines, while the guinea pig, monkey, and man are extremely sensitive to its absence or a low supply. Probably it is wholly a matter of different quantitative demands and therefore the rations mentioned may have supplied a sufficient quantity of this nutritive factor for the normal performance of such species as cattle, swine, or rats.

Antiscorbutic Properties of Sterilized Milk.

Frölich[10] and later Chick, Hume, and Skelton[11] have called attention to the poverty of heated milks in the antiscorbutic vitamine. Hess and Fish[12] and later Hess[13] reported a mild outbreak of infantile scurvy caused by the use, for several months, of a diet of cow's milk previously pasteurized at 63°C. for 30 minutes. The scorbutic symptoms disappeared on the restoration of the previously used antiscorbutic, orange juice, or on the substitution of raw milk for the heated milk.

[10] Frölich, T., *Z. Hyg. u. Infecktionskrankh.*, 1912, lxxii, 155.

[11] Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

[12] Hess, A. F., and Fish, M., *Am. J. Dis. Child.*, 1914, viii, 385.

[13] Hess, A. F., *Am. J. Dis. Child.*, 1916, xii, 152.

We have not as yet done any work with pasteurized milk, but have investigated, with respect to their antiscorbutic properties, milk sterilized at 120°C. for 10 minutes, two brands of unsweetened condensed milk, and one commercial brand of skimmed milk powder.

Milk sterilized at 120° for 10 minutes has lost its scorbutic protective powers for the guinea pig receiving a diet of rolled oats and dried hay (see Chart 16). A daily allowance per individual of 100 cc. of milk was made, but not all of this was consumed. The average daily consumption per individual, had it been raw milk, would have adequately protected them against scurvy. As it was, two of the animals died of scurvy at the end of 7 and 9 weeks respectively, after having consumed daily 47 cc. of milk per individual. The other two animals showed severe symptoms of the disease at the end of 4 weeks and had begun to lose weight. Their average daily milk consumption per individual had been 43 cc. At this point 1 cc. of orange juice per individual was administered daily by pipette with rapid recovery and gain in weight, demonstrating the adequacy of the diet in respect to all other factors but the antiscorbutic vitamine. The average daily milk consumption of these animals was much less during the orange juice-free period, but, including the orange juice-free period, the average milk consumption was 79 cc. per day per individual for the entire period of observation.

Antiscorbutic Properties of Unsweetened Condensed Milk.

The brands[14] of unsweetened condensed milk with which we have worked have also lost their antiscorbutic properties. The heat treatment given milks in the milk condensing industry varies considerably during different seasons of the year and also in different localities. A large condensing concern in reference to this matter of heat treatment states as follows:

"The general statement can be made that the pre-heating temperatures vary from 82.5°C. to 98.5°C. and the time from one minute to twenty minutes. The condensing temperature runs from 55°C. to 70°C., the time

[14] Golden Key, Valecia Evaporated Milk Co., Madison, Wis. Carnation, Carnation Milk Products Co., Seattle, Wash.

depending on the amount of milk condensed. The sterilizing temperatures run from 107°C. to 115.5°C., the time varying from twenty minutes to fifty minutes."

This is a more severe treatment than the sterilization of milk at 120°C. for 10 minutes. To test its antiscorbutic properties the condensed milk was diluted with an equal volume of water, making its content in solids equivalent to normal milk. A daily allowance to each animal of 100 cc. of this diluted milk was made, together with the usual ration of rolled oats and hay. The average daily milk consumption, had it been raw milk, would have amply protected against such an early attack of scurvy (see Chart 17). Two of the animals died from scurvy at the end of 3 and 5 weeks respectively. Their average daily consumption of milk per individual was 42 cc. The other two animals developed severe symptoms of the disease at the end of 5 weeks, at which time 1 cc. of orange juice per individual was administered by pipette daily. Rapid recovery followed, with pronounced increase in weight. The average daily consumption of milk per individual was 50 cc. for the entire period. While 30 cc. of whole raw milk on a rolled oats-hay diet offered protection against scurvy for 15 to 18 weeks, yet with these products, over 40 cc. per individual per day offered no protection whatever.

Antiscorbutic Properties of Milk Powder.

Judging from our limited data on milk powders accumulated up to the present time these products have likewise lost their antiscorbutic properties. Probably most of the milk powders on the market today are made by the spray process and here again there is no rigid uniformity among manufacturers as to time and heat treatments in the various stages for the process. One manufacturer informs us that in his plant the skimmed milk is heated for 20 minutes at 94°C. and then condensed in a vacuum from about five to one volume. The milk is next cooled rapidly and held in storage for the powder mill. The condensed milk is then run through a Jensen pasteurizer and heated to 60°C. just ahead of the hydraulic pump which forces the milk through the spray. The powder room is held at a temperature ranging from 70–77°C. The powder lies in this room during the day's run and is taken

out in the evening. Another manufacturer first condenses the milk and then pre-heats it to 49°C. and with the aid of a pump developing a pressure of 2,000 pounds per square inch, forces the spray into the powder room. A continuous current of hot air at an approximate temperature of 149°C. is forced into this room at a rate of 14,000 cubic feet per minute, which, when it comes in contact with the milk spray, immediately loses its high temperature of 140°C. and falls to 82°C. Gradually the temperature of 82°C. is reduced through the various channels of operation to a temperature of 55–65°C.

It is clear that the milk powders on the market have been exposed to heat treatments of varying degrees and probably in most cases of sufficient severity to destroy the antiscorbutic vitamine or at least greatly reduce its quantity. More data, however, are required to establish this point fully. In the light of our experience the report of the United States Public Health Service[15] on British experience in the use of milk powder in infant feeding is confusing. This report indicates a general success with milk powders in infant feeding, although it advises that "The occasional use of fruit juice is desirable."

In the records shown in Charts 18, 19, and 20 but a single brand[16] of milk powder was used. In our first experiments, Chart 18, 10 parts of the milk powder were mixed with 90 parts of rolled oats and the mixture was fed with the dried hay; the latter, as in all other experiments, was allowed in a separate container. Normal guinea pigs of 200 to 250 gm. in weight will consume daily 15 to 18 gm. of the oat-milk powder mixture. This amount of food, containing 10 per cent of milk powder, would mean the consumption of an equivalent in raw milk of but 15 to 18 cc. per day. All the animals developed scurvy and died in 4 to 5 weeks. The allowance of milk powder was insufficient to test adequately the problem in hand.

In our next experiment with the same powder (Chart 19) the milk powder constituted 25 per cent of the oat-milk powder mixture. With a similar daily consumption per individual of 15 to 18 gm. of the oat-milk powder mixture an equivalent of 40 to 45

[15] *Bull. Hyg. Lab., U. S. P. H., No. 473*, 1918.

[16] This brand was a Merrill Soule powder, concerning which no record of heat treatment could be secured.

cc. of raw milk would have been consumed. This quantity of raw milk would have been adequate for at least a long delay in the development of scurvy symptoms. Yet all these animals died with typical scurvy in 5 to 6 weeks.

In Lot 20, Chart 20, the proportion of skimmed milk powder was further increased and made 50 per cent of the oat-milk powder ration. With guinea pigs of 250 to 275 gm. in weight and a daily consumption of 15 to 18 gm. of the mixture per individual, there would be a daily consumption of milk powder equivalent to 75 to 90 cc. of raw skimmed milk. Yet on this liberal allowance, which as raw milk would have permanently protected against scurvy, all the animals succumbed to the disease in 5 to 15 weeks.

The lesson is obvious. Either the results with guinea pigs on experimental scurvy should not be translated to infantile scurvy, or we should follow the wiser course of using some antiscorbutic in conjunction with the exclusive use in infant feeding of such heated milk products as described in this paper.

SUMMARY.

1. On a diet of rolled oats and hay the prevention of scurvy by the use of raw milk will depend upon the amount of raw milk allowed. This confirms the results of Chick and her associates.

2. Such laxatives as mineral oil or phenolphthalein or the sugar, lactose, are not in themselves preventives of scurvy.

3. Milk sterilized at 120°C. for 10 minutes, commercial unsweetened condensed milk, and the commercial milk powder examined had lost their antiscorbutic properties when used in quantities equivalent to an amount of raw milk which would prevent scurvy in guinea pigs on a diet of rolled oats and dried hay.

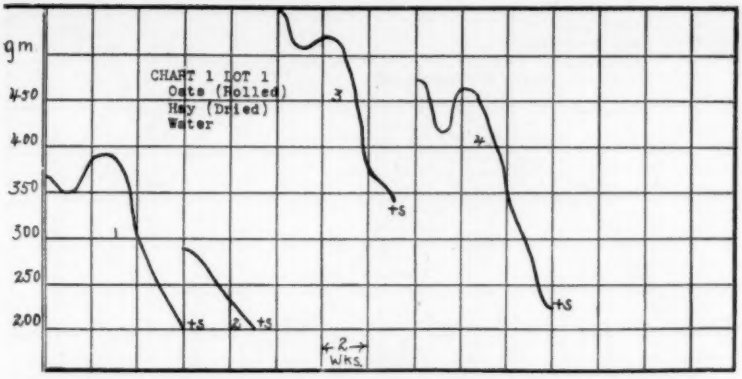

CHART 1. Lot 1 received a diet of rolled oats and dried hay (a mixture of timothy and June grass). These materials were fed separately, the hay being put in the cage uncut. The hay was eaten with a relish. This ration provided all the factors of nutrition, except a good protein mixture and the antiscorbutic factor. Guinea pigs invariably died of scurvy on such a ration.

S = Scurvy.

\+ = Died.

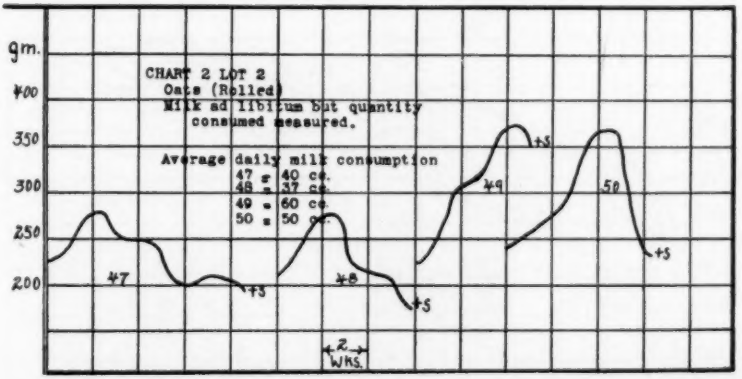

CHART 2. All the animals in Lot 2 died of scurvy. Without the hay and with nothing but rolled oats and milk *ad libitum* (actual consumption of milk shown in chart) they were unable to survive. The quantity of whole milk consumed was undoubtedly too small to act as an efficient scorbutic protector.

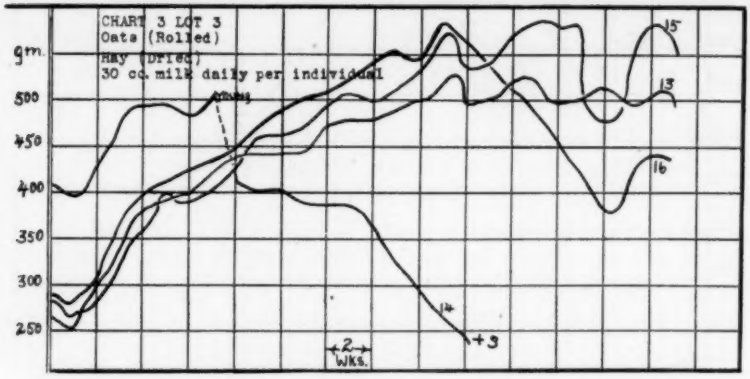

CHART 3. This lot received rolled oats, dried hay, and a small allowance per individual of fresh whole milk. By a daily consumption of 30 cc. of fresh whole milk per individual, there was great delay in the onset of scurvy. The hay probably contributed some of the necessary antiscorbutic, in addition to roughage, salts, and fat-soluble vitamine, and through this improvement in the ration over a rolled oats-milk diet made possible an effective protection against scurvy with a small supply of milk. Animals 13, 15, and 16 showed enlargement of the joints at the end of 20 weeks, indicating a mild condition of scurvy.

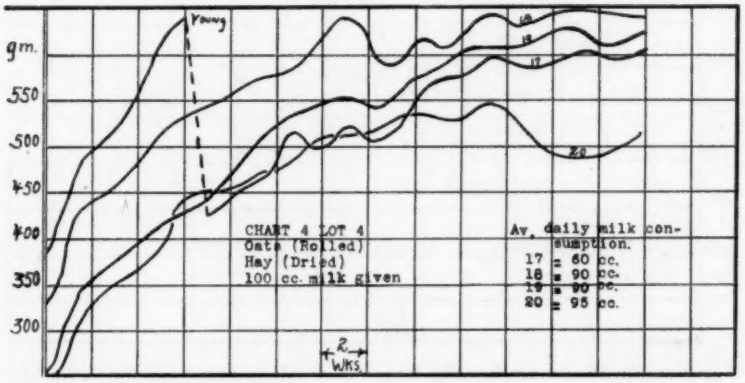

CHART 4. Complete protection against scurvy on a rolled oats-dried hay diet when the fresh whole milk consumed daily per individual equaled 60 cc. or more.

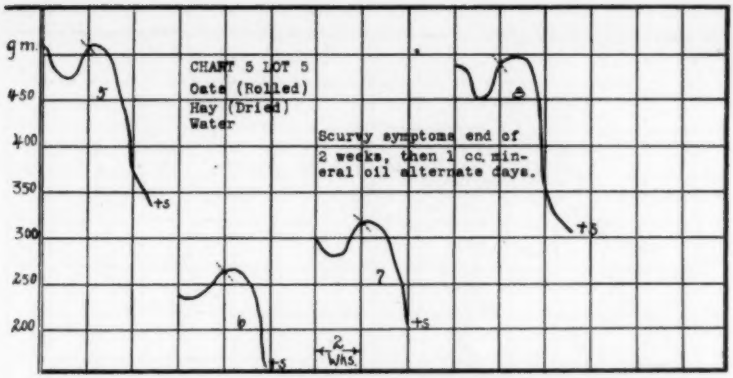

CHART 5. On a diet of rolled oats and dried hay mineral oil did not protect the guinea pig against the course of scurvy after it had developed. The theory that scurvy is primarily related to intestinal disorders is untenable.

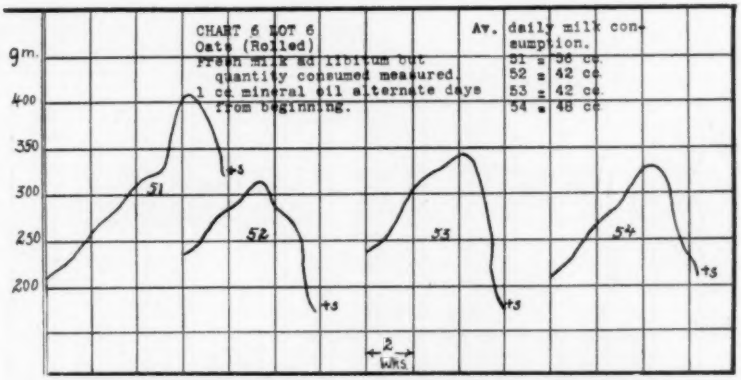

CHART 6. The behavior of this lot is further evidence of the non-protection against scurvy by the use of a laxative such as mineral oil. A diet of rolled oats and whole milk (with an average daily consumption per individual of 42 to 56 cc.) produced scurvy even with an administration on alternate days of 1 cc. of mineral oil per individual. The milk addition improved the ration for growth. Where cures or preventions of scurvy by the use of a laxative have been accomplished on a diet of rolled oats and milk *ad libitum*, such as reported by McCollum and Pitz,[6] it is probable that a larger consumption of milk occurred than was here observed.

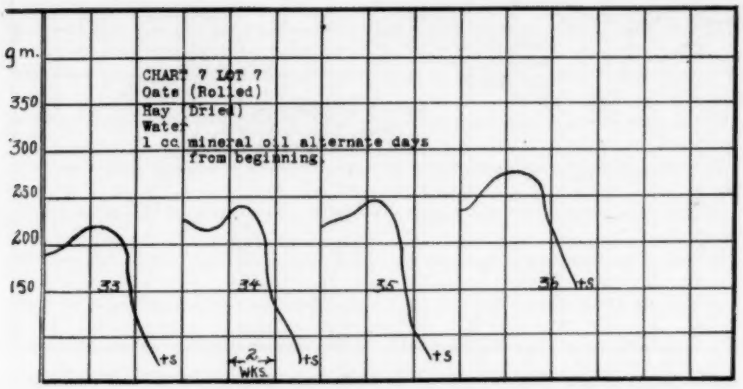

CHART 7. Administration of the mineral oil from the time the animals were placed on the diet of oats and hay was equally ineffective in protection against scurvy (cf. Chart 5).

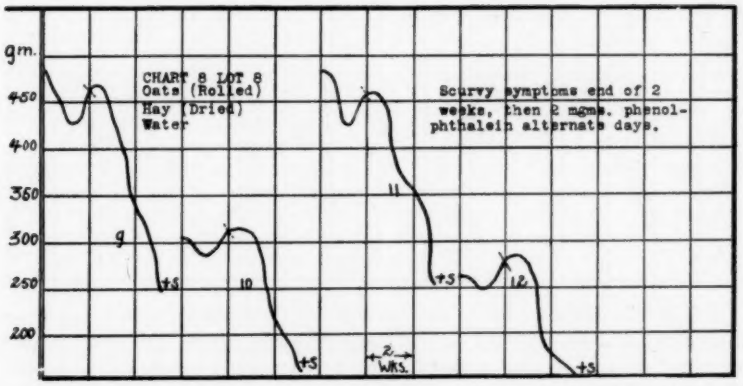

CHART 8. Phenolphthalein was ineffective as an antiscorbutic with guinea pigs receiving a diet of oats and dried hay. Administration of the phenolphthalein was begun after the early symptoms of scurvy appeared.

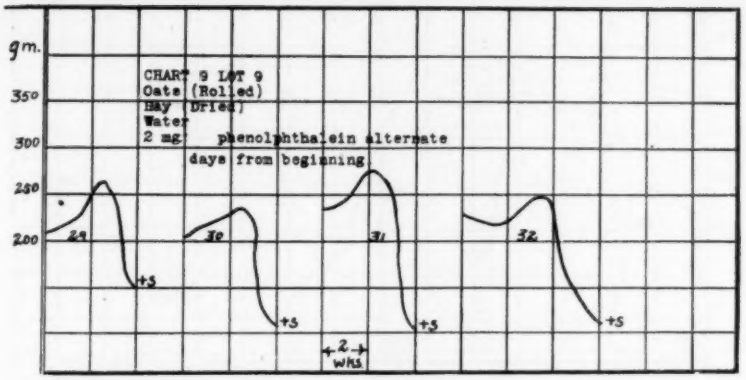

CHART 9. Administration of phenolphthalein on alternate days from the beginning of the experiment failed to protect guinea pigs, receiving a diet of rolled oats and dried hay, against the development of scurvy.

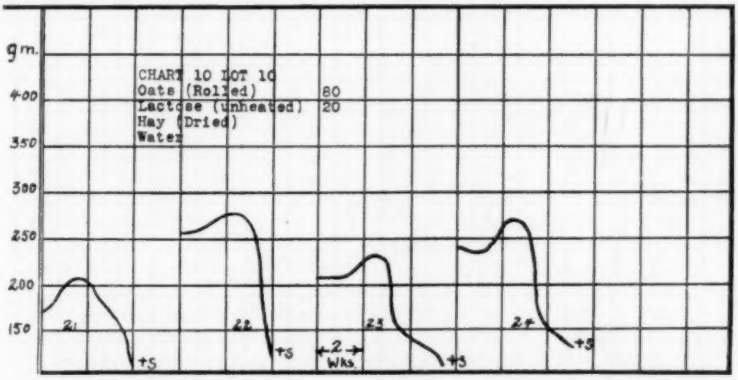

CHART 10. This Chart illustrates the record of guinea pigs receiving a diet of rolled oats, dried hay, and unheated lactose. They all died with scurvy. On a diet of rolled oats, salt mixture, and milk, Pitz[9] had succeeded in dispelling the symptoms of scurvy by the use of lactose. The probable explanation of that result lies in a larger milk consumption by the animals after lactose administration. This would then mean that recovery was really due to an increased intake of the antiscorbutic vitamine.

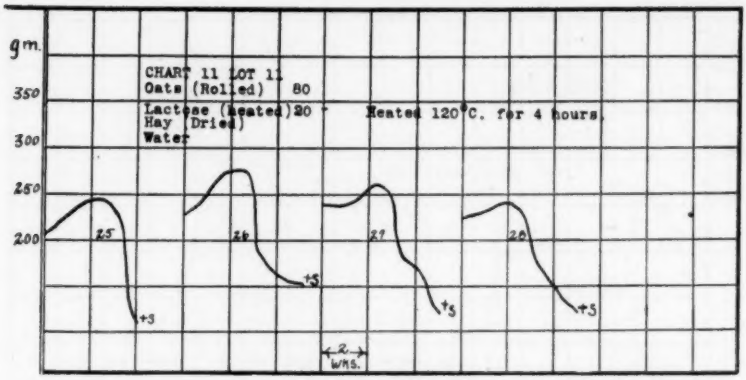

CHART 11. Lactose heated for 4 hours at 120°C., to destroy any antiscorbutic factor that it might contain, differed in no way from unheated lactose in its effects on the development of scurvy on a diet of rolled oats and dried hay.

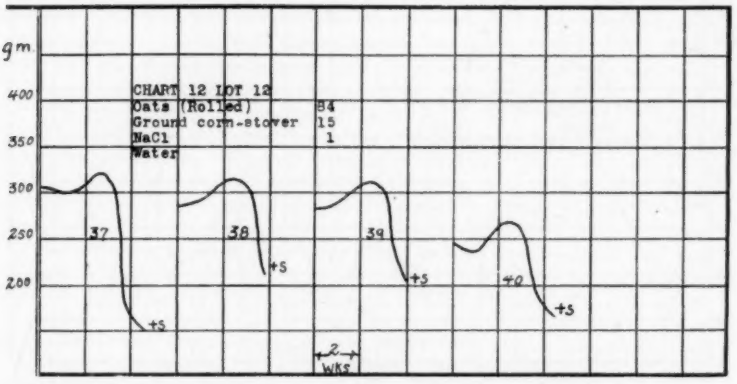

CHART 12. This Chart illustrates how a diet, satisfactory for one species, will fail with another. Cattle or swine could be reared at a slow rate of growth and maintained on a ration of rolled oats 84 parts, corn-stover 15 parts, and common salt 1 part, but this dried material is too poor in the antiscorbutic to prevent scurvy in the guinea pig. This may mean only that cattle and swine demand less of the antiscorbutic factor than do guinea pigs.

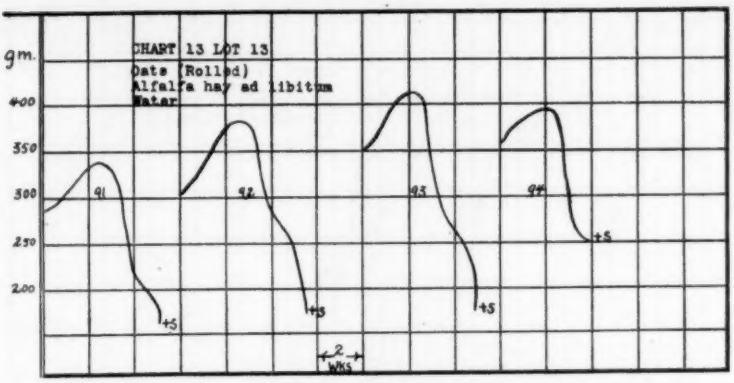

CHART 13. Another illustration of the failure of a dried roughage, such as alfalfa, to protect against scurvy in the guinea pig. A ration of rolled oats and dried alfalfa would be adequate for swine, cattle, or rats, as far as maintenance is concerned, although it would not allow normal growth in swine or rats; but with the guinea pig it leads to scurvy. This is interpreted as a difference in the antiscorbutic demands of the species and as an indication that the rolled oats and dried alfalfa have lost much of their antiscorbutic vitamine in drying or aging.

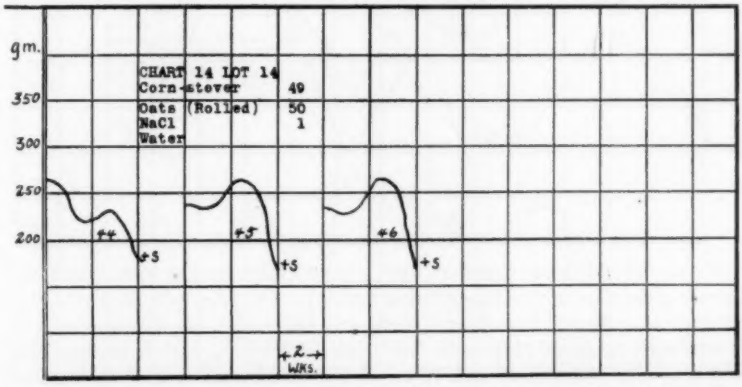

CHART 14. Even a larger proportion of dried corn-stover in the diet (49 per cent as contrasted with 15 per cent, see Chart 12) will not protect the guinea pig against scurvy. The roughages, such as alfalfa or corn-stover, that were used in these experiments were the ordinary dried material stored in the University barn from the crop of 1918.

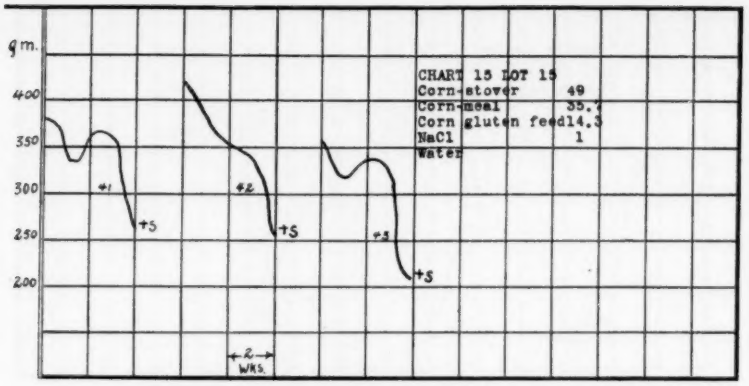

CHART 15. A ration which has grown and maintained cattle for 4 years as the sole diet, but which produced scurvy in the guinea pig. Either cattle are not susceptible to the disease or their demands for the anti-scorbutic vitamine are relatively less.

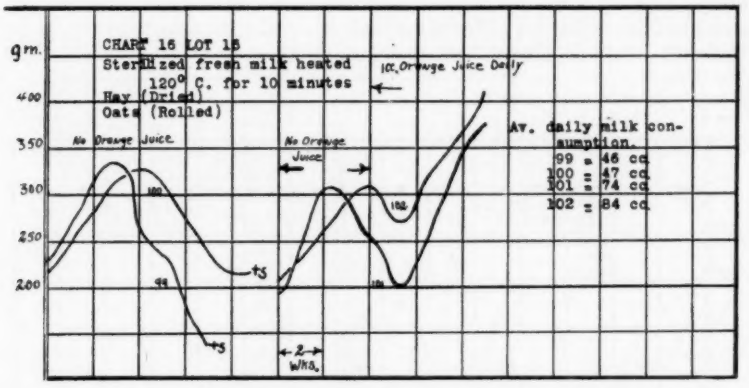

CHART 16. Milk sterilized at 120°C. for 10 minutes has lost or suffered a great reduction in its antiscorbutic content. On a diet of rolled oats, hay (dried), and 30 cc. of fresh milk daily per individual the development of scurvy can be greatly delayed (see Chart 3). Yet, with a similar diet and a consumption of 46 and 47 cc. of sterilized milk scurvy terminated the life of Animals 99 and 100 in 7 to 9 weeks. The curves of Animals 101 and 102 illustrate the recovery from scurvy brought about by a diet of rolled oats, hay, and sterilized milk, but with the daily administration of 1 cc. of orange juice per individual. After the administration of the orange juice and recovery the average milk consumption per day was considerably increased.

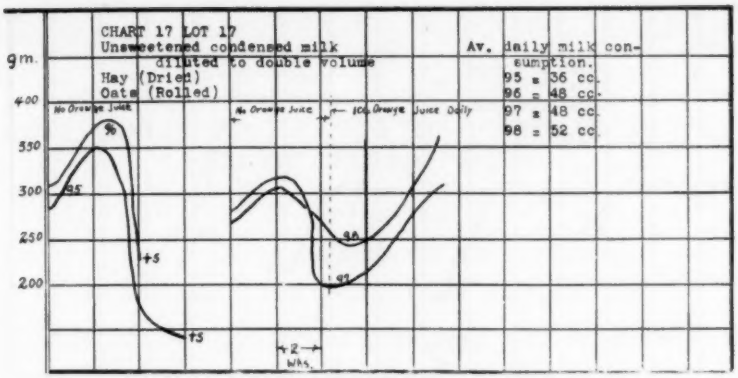

CHART 17. The brands of unsweetened condensed milk used in these experiments had also lost their antiscorbutic properties. The consumption of this milk, diluted so as to be equivalent in concentration to normal fresh milk, was 36 to 52 cc. per individual per day. All developed scurvy in 3 weeks and the disease was allowed to run its course in the cases of Animals 95 and 96. To the other two animals 1 cc. of orange juice was given daily by pipette with complete recovery, except for the permanent enlargement of the joints.

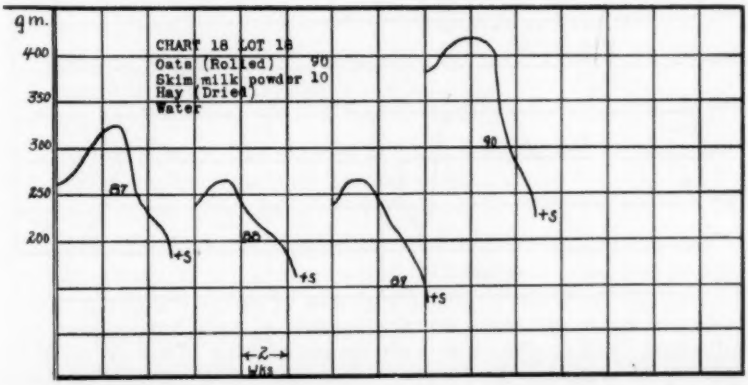

CHART 18. The brand of commercial milk powder examined possessed no protective qualities against scurvy. On a diet of rolled oats, dried hay, and a daily allowance of milk powder, equivalent to 15 to 18 cc. of skim milk per individual, scurvy terminated the life of the animals in 5 weeks.

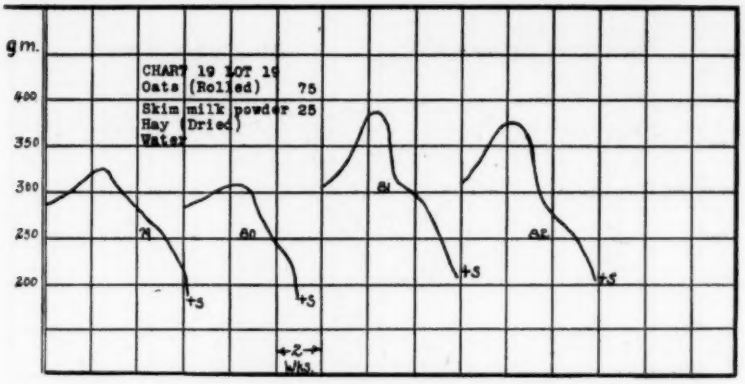

CHART 19. Another failure on milk powder. When the allowance of milk powder was increased to an amount equivalent to 40 to 45 cc. of raw milk per individual per day, with a diet of rolled oats and hay, scurvy rapidly developed and all of this group died of the disease in 5 to 6 weeks.

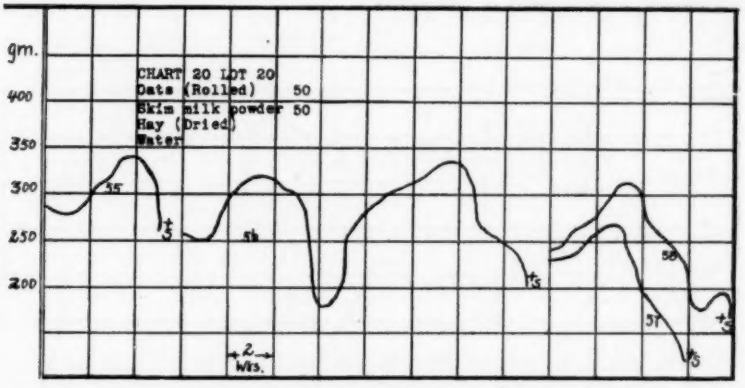

CHART 20. A still more liberal allowance of milk powder than was made with the lots whose curves are shown in Charts 18 and 19, failed to prevent the development of scurvy. The milk powder allowed per individual per day was equivalent to 75 to 90 cc. of fresh skim milk. Three of the animals rapidly succumbed to the disease, lasting but 5 to 7 weeks. Animal 56 developed scurvy as early as the other three in the lot, but in spite of the progress of the disease it started to regain in weight after the first period of 6 weeks, but finally died of scurvy at the end of 15 weeks.

NUTRITIONAL STUDIES ON THE GROWTH OF FROG LARVÆ (RANA PIPIENS).

First Paper.

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Plates 2 to 5.

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A number of studies have been reported upon the growth and development of frog larvæ as influenced by feeding certain products *in toto*, such as glandular materials. As far as we are aware, however, no one has incorporated in the feeding of such larvæ conditions which were intended to control definitely the nutritive value of the diet in terms of protein, carbohydrate, fat, mineral salts, and the food accessories, hormones or vitamines. We have, therefore, attempted in this preliminary report to give a summary of our studies along this line with a view of supplementing it from time to time in the light of further findings.

Hopkins, Osborne and Mendel, McCollum and associates, and several others have made, during the last few years, valuable contributions, in their studies with rats and mice, to the nutritive value of synthetic food mixtures. They have shown that proteins, fats, salt mixtures, and vitamines each occupy a peculiar place in the physiological economy—not alone in respect to the amount present in diet but also in respect to the quality or kind of each nutrient.

It was thought by using this particular species of amphibians, namely frog larvæ (*Rana pipiens*) which have a characteristic life cycle—passing through the tadpole stage to the frog, that we might be able to determine some interesting facts regarding their nutrition with respect to size and degree of metamorphosis. One great advantage in using such larvæ in place of larger animals

is that one can work with large numbers and thereby be able to draw fairly definite conclusions. On the other hand, it is readily seen that serious objections may be raised in the making of practical tests. For example, if the larvæ are fed in large groups there is no way of measuring the food intake, or of preventing the more vigorous ones from tāking the food from the weak, or of eating the tadpoles that die occasionally, and no means of controlling the water-soluble content of the diet if water is allowed to flow through the trays continuously.

EXPERIMENTAL.

The tadpole eggs were collected in the near vicinity and brought to the laboratory with every precaution to prevent injury, since in the late stages of segmentation the eggs are very delicate. Immediately after hatching, the tadpoles were grouped into colonies of 500 each, being placed in enamel-lined trays. Special attention was paid to the hygienic conditions of the surroundings. Thus, the ventilation of the room, which was used only for this work, was regulated; the bright light was eliminated; the trays were thoroughly cleaned at regular intervals; unused food was removed frequently to prevent the formation of toxic substance due to decomposition; and fresh water was supplied two to three times daily. The water used was from the city supply but it was first passed through a Hygeia filter which removed the suspended organic matter.

In order to obtain a permanent record of the progress of growth, photographs were taken each week of a selected group from each colony. The selection was governed by choosing from each tray an equal number of the largest, medium, and smallest specimens. Shadow photographs were made by subjecting the tadpoles to an instantaneous exposure of an arc light. From these photographic records, measurements were made of all the tadpoles, including body width, body length, and total length with tail.

Late in the experiment when the limb buds began to appear, microscopic examination of all the tadpoles in each tray was made. Following a definite scheme, each tadpole was classified according to its stage of development and from these data the

percentage distribution of the tadpoles was calculated for the groups that were on different diets. In addition to this an attempt was made to preserve every week representative specimens from every group and to make a histological study of the serial sections made from these specimens. The individual variations between the tadpoles proved to be too great a factor and for this reason the histological data will not be reported at this time. Text-fig. 1, page 333, illustrates the scheme followed in determining the various stages of development of the hind legs of the frog larvæ.

The feeding of the colonies or groups was done at specified times, always following the cleaning of the trays. The endeavor was to feed enough yet not too much, so as to avoid an excess of material remaining in the trays. This was governed in part by weighing out the food.

The synthetic diets were made up of various combinations of food substances: for protein, lactalbumin (1) and corn gluten (2) were used; cystine was also introduced as a supplement to corn gluten; for carbohydrate, purified starch or dextrin was used, and lactose, where Osborne and Mendel's protein-free milk (3) was incorporated; for fat, butter fat was employed as a carrier of fat-soluble A, lard was also used; for mineral salts, the McCollum Salt Mixture 185 was taken (4) except when protein-free milk was in the diet; for water-soluble B vitamine, either an alcohol extract of dried brewers' yeast, or protein-free milk was selected; and for another source of fat-soluble A, other than butter fat, we took an alcohol extract of linseed oil meal (5) which had first been treated with ether to remove the oil. Table I gives the composition of the diets fed.

DISCUSSION.

I. Effect of Diet on the Size of the Tadpole.

(*A*) *Influence of the Amount of Fat.*—In Table I, it will be seen that the rations or diets were arranged in two general classes, Groups I and II. The former was made up in accordance with the usual formula proposed by Osborne and Mendel; that is, these diets were high in fat, containing from 23 to 28 per cent of

TABLE I.

*Percentage Composition of the Diets.**

Diet No.	Foods in diet.	per cent	Diet No.	Foods in diet.	per cent
I_1	Lactalbumin (protein).	10.0	II_1	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Butter fat.	18.0		Butter fat.	5.0
	Lard.	10.0		Yeast extract (alcoholic.)	1.0
I_2	Lactalbumin (protein).	10.0	II_2	Lactalbumin (protein).	10.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	18.0		Butter fat.	5.0
	Lard.	10.0			
I_3	Lactalbumin (protein).	10.0	II_3	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Lard.	28.0		Yeast extract (alcoholic).	1.0
I_4	Lactalbumin (protein).	10.0	II_4	Lactalbumin (protein).	10.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Lard.	28.0		Lard.	5.0
I_7	Lactalbumin (protein).	10.0	II_7	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Linseed meal extract (alcoholic).	5.0		Linseed meal extract (alcoholic).	5.0
	Lard.	23.0		Yeast extract (alcoholic).	1.0
				Lard.	5.0
I_9	Lactalbumin (protein).	10.0	II_9	Lactalbumin (protein).	10.0
	Protein-free milk, purified.	28.0		Salt mixture.†	3.7
	Yeast extract (alcoholic).	1.0		Yeast extract (alcoholic).	1.0
	Lard.	27.0		Lard.	5.0
III_5	Corn gluten (protein).	10.0	III_4	Corn gluten (protein).	10.0
	Salt mixture.†	3.7		Cystine.	0.2
	Butter fat.	5.0		Salt mixture.†	3.7
	Yeast extract (alcoholic).	1.0		Butter fat.	5.0
				Yeast extract (alcoholic).	1.0

TABLE I.—*Concluded.*

Diet No.	Foods in diet.		Diet No.	Foods in diet.	
		per cent			*per cent*
II_{1a}	Lactalbumin (protein).	30.0	II_{2a}	Lactalbumin (protein).	30.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Butter fat.	5.0
	Yeast extract (alcoholic).	1 0			
IV_4	Lactalbumin (protein).	15.0	IV_5	Lactalbumin (protein).	15.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Butter fat.	5.0
	Yeast extract (alcoholic).	1.0			
IV_6	Lactalbumin (protein).	15.0	IV_8	Lactalbumin (protein).	15.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Lard.	5.0		Yeast extract (alcoholic).	1.0
IV_{10}	Beef tissue (protein).	15.8	V_1	Lactalbumin (protein).	5.0
	Oats, rolled (protein).	1.9		Corn gluten (protein).	5.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Butter fat.	5.0
	Yeast extract (alcoholic).	1.0		Yeast extract (alcoholic).	1.0
III_6	Lactalbumin (protein).	10.0	VI_2	Lactalbumin (protein).	30.0
	Salt mixture.†	3.7		Salt mixture.†	3.7
	Butter fat.	5.0		Lard.	5.0
	Yeast extract (alcoholic).	1.0			

* In these rations purified starch was used in making up the total percentage, except in Ration III_6 where dextrin was employed.

† McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105. Salt Mixture 185.

either lard alone or a mixture of lard and butter fat. In group II, the McCollum formula was followed as to fat; *i.e.*, 5 per cent.

It will be seen from Fig. 1 that the tadpoles fed the high fat diet (I_1) were smaller than those that were fed the low fat diet (II_1). The periods referred to were 1 week apart. These permanent records were given from the time that differences began

to be evident. The diets in these two cases were complete in the sense that they were sufficiently well balanced as to all the essential nutrients needed to produce normal growth in young rats.

(*B*) *Influence of the Vitamines.*—Inasmuch as the low fat diet appeared to be best for the tadpole, it is obvious that any comparisons between groups with respect to their nutritive value should be made on this plane. If the diets from Groups II_1 and II_2 are compared, it will be seen that they differed in that the yeast vitamine was removed from the former and an equivalent amount of starch substituted for it. In Fig. 2, the control Group II_1 showed distinctly larger tadpoles than Group II_2, up to Period 10, when the differences were not nearly so marked. Whether this convergence was due to the fact that the diet for Group II_1 may have lacked some essential constituent which retarded a more rapid growth should be borne in mind. It is evident that the water-soluble B was essential up to a certain stage in the growth. Beyond this it may be that the tadpole was able to store enough of this form of vitamine in the tail to supply its needs, just as certain bacteria appear to need vitamines at the start but later they synthesize the vitamines themselves (6).

Ration II_9 was deficient in fat-soluble A vitamines but in other respects it corresponded to Ration II_1, lard being substituted for butter fat. From Fig. 3 it is evident that the tadpoles fed Ration II_1 grew better than those that lacked the fat-soluble A. After the eleventh period, however, the differences were too slight to be significant. In other words, the tadpoles seemed to need the fat-soluble accessory more in the early stages of growth, while later on there was an adaption, or a reserve supply furnished through the medium of the tail.

When both vitamines were withdrawn from the diet, the tadpoles did not grow so vigorously. Contrary to what we have found with rats, however, they did show a gradual rate of growth. This may have been due in part to their ability to draw on the reserve stored in the tail, for in each instance the tail length was much shorter than with the tadpoles fed Ration II_1. In all cases, though, the body length and body width were also less, so that there was a general reduction in body size. Figs. 4 and 5 illustrate this. In the former case, the diet was based on a 10 per cent protein plane and in the latter case on a 30 per cent protein

plane. The differences were more marked on the higher protein diet (Fig. 5).

(*C*) *Effect of the Amount of Protein.*—Osborne and Mendel (7) and Emmett and Luros (8) have been able to obtain normal growth with rats with 10 per cent of lactalbumin protein. Osborne and Mendel found that it required 50 per cent more casein and 90 per cent more edestin than lactalbumin to bring about normal growth in rats. Wheeler (9) in working with mice determined that the best results for growth were obtained when the casein was as high as 30 per cent. It might therefore be that while lactalbumin is one of the very best proteins for promoting growth, the quantity needed for tadpoles should be higher than for rats.

Fig. 6 shows that when the protein plane was 30 per cent (II_{1a}) the tadpoles grew more rapidly than when it was 10 per cent (II_1). In the former case the body width, length, and total length were greater than for Group II_1. It should be stated that Group II_{1a} was obtained by subdividing Group II_1 in Period 9.

(*D*) *Effect of the Kind of Carbohydrate.*—In the large majority of the low fat diets, starch which had been purified by washing with hot 95 per cent alcohol was used as the sole source of carbohydrate. McCollum used dextrin in many of his rations and since the low fat diets were based essentially upon his formula, we incorporated dextrin in Ration III_6 to determine whether it was an essential factor and would accelerate growth. The comparative value of the two rations, II_1 and III_6,—the former starch and the latter dextrin—is shown in Fig. 7. There was practically no difference in the size of the two groups of tadpoles.

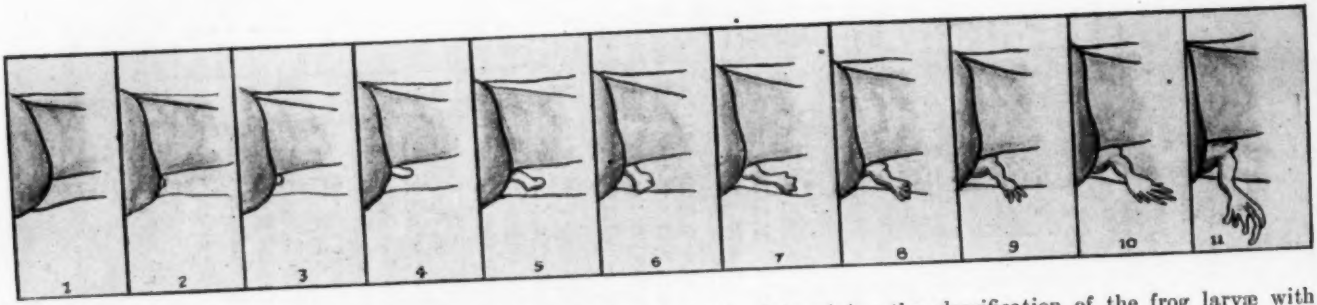
(*E*) *Value of Beef and Oat Diet.*—It is known that tadpoles will thrive on a diet of beef and oats. We therefore made up a control diet which was the same as Diet II_1 with the exception that we used 15.8 per cent of desiccated ether-extracted beef tissue protein and 1.9 per cent of ether-extracted rolled oats protein. Fig. 8 shows that we obtained our optimum growth with this ration. That this result was not due to the large amount of protein is evident, for when Fig 6, which shows tadpoles that were fed 30 per cent protein, is compared with Fig. 8, the beef-oats diet was the best although it contained 17.7 per cent protein.

To what this difference was due, we are not as yet able to state. However, in comparing the two rations II_1 and IV_{10}, it is evident that the beef tissue and rolled oats were the contributing factors; and that the presence of the butter fat, yeast extract, starch, and salt mixture in the diet did not inhibit growth. McCollum, Simmonds, and Parsons (10) claim that lactalbumin is an incomplete protein for growth. Emmett and Luros (8) found under certain conditions that they were not able to obtain good growth with lactalbumin but that the apparent trouble lay in the fact that this protein was either susceptible to toxic substances or else it needed the presence of a water-soluble vitamine other than the water-soluble B. When lactose or the protein-free milk of Osborne and Mendel was present, normal growth was obtained. It would appear that perhaps a similar condition of affairs took place with the tadpoles. This question is now being investigated.

II. Effect of Diet on Development of Hind Legs of Tadpoles.

Growth and development may bear only a limited relation to each other in tadpoles maintained in an artificial environment. To state that a specimen of certain measurements is in a corresponding stage of metamorphosis may, upon closer inspection, prove untrue. Individual variation in both respects has been found by us to be marked. Consequently the colonies should be fairly large and observation should be made on as many members as possible in order that the true average may be most nearly approached. Bearing this in mind, all the tadpoles in each of the groups were examined as to the stage of development. This, as was previously stated, was not carried out in the observations made for growth or size.

(*A*) *Influence of the Amount of Fat in the Diet.*—In Table II are presented the data giving the percentage distribution of the tadpoles in each group with respect to the stage of development of the hind legs. These data, for two of the experimental periods (Nos. 12 and 14), near the close of the series of tests, are arranged chiefly with reference to the amount of fat in the diet, and also the kind of vitamine that is present. The detailed percentage composition of the diets is given in Table I. It is to be noted



TEXT-FIG. 1. The above drawings illustrate the scheme followed in determining the classification of the frog larvæ with respect to the development of the hind legs—as used in Tables II, III, and IV.

TABLE II.

Influence of Amount of Fat upon Development of Hind Legs of Rana pipiens Larvæ.

Percentage Distribution within Groups.

Group.	Diet.		Experimental period.	Stages of development of hind legs.*										
	Class of vitamines.	Fat.		1	2	3	4	5	6	7	8	9	10	11
		per cent												
I_1	Water- and	28†	12	11.1	62.9	22.9	6.3	—	—	—	—	—	—	—
I_1	fat-soluble.	28†	14	—	45.4	36.3	18.2	—	—	—	—	—	—	—
II_1	" " "	5‡	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—
II_1	" " "	5‡	14	—	14.6	34.1	29.5	7.3	2.4	—	4.9	2.4	4.9	—
I_7	Water- and	23§	12	8.1	43.9	40.0	8.1	—	—	—	—	—	—	—
I_7	fat-soluble.	23§	14	6.6	50.4	32.4	10.4	—	—	—	—	—	—	—
II_7	" " "	5§	12	9.1	35.6	37.4	14.1	1.1	1.1	1.1	—	—	—	0.5
II_7	" " "	5§	14	8.6	16.8	47.0	21.6	3.6	—	1.2	1.2	—	—	—
I_2	Fat-soluble.	28†	12	31.3	43.4	20.2	5.1	—	—					
I_2	"	28†	14	28.3	35.8	25.0	10.9	—	—					
II_2	"	5‡	12	9.4	40.5	36.5	12.2	1.4	—					
II_2	"	5‡	14	—	20.3	53.6	17.2	7.8	1.6					
I_3	Water-sol-	28§	12	11.4	56.1	26.5	5.3	0.8	—	—	—	—	—	—
I_3	uble.	28§	14	13.7	34.4	41.0	10.7	—	—	—	—	—	—	—
II_3	"	None.	12	1.5	12.1	34.8	24.2	15.1	9.1	—	1.5	—	—	1.5
II_3	"	"	14	3.3	13.1	18.0	41.0	8.2	3.3	8.2	3.3	1.6	—	—
I_9	Water-sol-	27§	12	—	65.3	26.5	8.2	—						
I_9	uble.	27§	14	—	68.9	17.3	13.8	—						
II_9	"	5§	12	23.9	51.4	17.4	6.5	0.7						
II_9	"	5§	14	17.6	30.4	39.2	10.8	1.9						
I_4	None.	28§	12	11.2	46.5	33.6	8.6	—						
I_4	"	28§	14	10.2	35.1	32.4	21.3	0.9						
II_4	"	5§	12	17.0	53.2	19.5	7.3	—						
II_4	"	5§	14	16.6	39.0	28.0	16.6	—						

* See Text-fig. 1, p. 333.

† 18 per cent butter fat and 10 per cent lard.

‡ Butter fat.

§ Lard

that the group number and diet number correspond. Thus, in Table II, Group I_1 was fed Diet I_1.

If Group I_1 (28 per cent fat) is compared with Group II_1 (5 per cent fat) it is seen, when both the water-soluble B and fat-soluble A accessory substances were present, that the tadpoles fed the low fat diet developed much better. In Groups I_7 and II_7, the fat-soluble A was supplied by an alcoholic extract of linseed meal, whereas it was furnished in the two preceding groups by butter fat. The difference between these two groups was clearly in favor of the low fat diet. With Groups II_1 and II_7, we have a comparison of two kinds of fat, butter fat and lard, and it appears that 5 per cent of either was permissible for fair development. Whether a smaller amount would have been much better is being tried out.

When the diet lacked the water-soluble B vitamine and contained the fat-soluble A in the form of butter fat, as shown in Groups I_2 and II_2, the low fat diet produced the best results. The differences were not so marked, however, as in the cases of Diets I_1, I_7, II_1, and II_7.

Groups I_3 and II_3 were fed diets which were deficient in fat-soluble A. In the case of the latter group no fat was used. It will be seen that this group was far in advance of the high fat-fed colony. In fact, there is such a marked contrast that the question immediately arises as to what extent Group II_1 would have developed if we had used some other source of fat-soluble A than a fat, as butter fat, and what results would have been obtained if the lard had been omitted from Diet II_7. In Groups I_9 and II_9, this point is partially answered since the diet for II_9 differed only from that of II_3 in having 5 per cent lard added to it and in having a corresponding decrease in starch. It would appear in comparing Groups II_9 and II_3 that the fat had a marked inhibiting effect. With respect to Groups I_9 and II_9, which contained 27 and 5 per cent of lard respectively, there was only a slight difference in favor of the lower fat diet.

When both vitamines were absent, as in the Groups I_4 and II_4, the high fat diet appeared to produce somewhat better results than the low fat diet.

It is evident from these data that the amount of fat in the diet had an appreciable influence upon the development of the tad-

TABLE III.

Influence of Vitamines upon Development of Hind Legs in Rana pipiens Larvæ. Percentage Distribution within Groups.

Group.	Diet.		Experimental period.	Stages of development of hind legs.*										
	Class of vitamines.	Fat.		1	2	3	4	5	6	7	8	9	10	11
		per cent												
I_1	Water- and fat-soluble.	28†	12	11.1	62.9	22.9	6.3	—						
I_1	" " "	28†	14	—	45.4	36.4	18.2	—						
I_7	" " "	23‡	12	8.1	43.9	40.0	8.1	—						
I_7	" " "	23‡	14	6.7	50.5	32.4	10.4	—						
I_2	Fat-soluble.	28†	12	31.3	43.4	20.2	5.1	—						
I_2	"	28†	14	28.2	35.9	25.0	10.9	—						
I_3	Water-soluble.	28‡	12	11.4	56.1	26.5	5.3	0.8						
I_3	"	28‡	14	13.7	34.3	41.2	10.8	—						
I_9	"	28‡	12	—	65.3	26.5	8.2	—						
I_9	"	28‡	14	—	68.9	17.3	13.8	—						
I_4	None.	28‡	12	11.2	46.5	33.6	8.6	—						
I_4	"	28‡	14	10.2	35.2	32.4	21.3	0.9						
II_1	Water- and fat-soluble.	5§	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—
II_1	" " "	5§	14	—	14.6	34.1	29.5	7.3	2.4	—	4.9	2.4	4.9	—
II_7	" " "	5‡	12	9.1	35.6	37.4	14.1	1.1	1.1	1.1	—	—	—	0.5
II_7	" " "	5‡	14	8.6	16.8	47.0	21.6	3.6	—	1.2	1.2	—	—	—
II_2	Fat-soluble.	5§	12	9.4	40.5	36.5	12.2	1.4	—	—	—	—	—	—
II_2	"	5§	14	—	20.3	53.1	17.2	7.8	1.6	—	—	—	—	—
II_3	Water-soluble.	None.	12	1.5	12.1	34.8	24.2	15.1	9.1	—	1.5	—	—	1.5
II_3	"	"	14	3.3	13.1	18.0	41.0	8.2	3.3	8.2	3.3	1.6	—	—
II_9	"	5‡	12	23.9	51.4	17.4	6.5	0.7	—	—	—	—	—	—
II_9	"	5‡	14	17.6	30.4	39.2	10.8	1.9	—	—	—	—	—	—
II_4	None.	5‡	12	17.0	53.2	19.5	7.3	—	—	—	—	—	—	—
II_4	"	5‡	14	16.6	39.0	27.8	16.6	—	—	—	—	—	—	—

* See Text-fig. 1, p. 333.

† 18 per cent butter fat and 10 per cent lard.

‡ Lard.

§ Butter fat.

pole; *i.e.*, the higher the fat content, the lower the stage of development.

(*B*) *Influence of Vitamines.*—In Table III, the data for the percentage distribution of the tadpoles within the groups are arranged with respect to the presence or absence of vitamines in the diet. The upper half of the table gives the results for the high fat diets. It would appear that the excess of fat was such a large factor that there was practically no difference between the groups.

In regard to the low fat diet Group II_1 was somewhat further advanced than Group II_7. This may have been due to the lard or to the fact that the fat-soluble A accessory was supplied either qualitatively or quantitatively to a lesser degree than in II_1. When Groups II_1, II_2, and II_9 are compared, the withdrawal of either the water-soluble B (II_2) or of the fat-soluble A (II_9) resulted in a distinct inhibition or retardation in the development of the tadpoles. In the case of Group II_3, two factors seemed to have played a part, the lack of both fat-soluble A and of any fat. As a result, this group developed at practically the same rate as Groups II_1 and II_7. This would tend to suggest that the water-soluble B vitamine was more essential than the fat-soluble A, or that the tadpoles had a reserve supply of this latter accessory stored in the tail, upon which they could draw. When both vitamines were removed from the diet, however, it appears from Group II_4, that there was little or no reserve vitamine supply available, for these tadpoles developed more slowly than any of the other groups.

The data indicate that both vitamines are essential to the development of the tadpole; that when fat was present to the extent of 5 per cent and the fat-soluble A was absent, there was a marked suppression, but if there was no fat present, the rate of development appeared to be normal when compared with the groups fed the control diets (II_1 and II_7). When the water-soluble B vitamine was absent the cessation of the development of the tadpoles was very noticeable.

(*C*) *Influence of the Amount and Kind of Protein.*—The data in Table IV are so arranged that the values can be compared with respect to the amount of protein. There is sufficient information given to indicate the vitamines and also the amount and kind

TABLE IV.

Influence of Amount and Kind of Protein upon Development of Hind Legs of Rana pipiens Larvæ. Percentage Distribution within Groups.

Group.	Diet.				Experimental period.	Stages of development of hind legs.*										
	Protein.		Class of vitamines.	Fat.		1	2	3	4	5	6	7	8	9	10	11
	Kind.															
		per cent		*per cent*												
II_1	Lactalbumin.	10	Water- and fat soluble.	5†	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—
II_1	"	10	" " "	5†	14	—	14.6	34.7	29.5	7.4	2.4	—	4.9	2.4	4.9	—
IV_4	"	15	" " "	5†	12	5.1	21.4	21.4	26.6	15.2	3.8	2.5	1.3	—	2.5	—
IV_4	"	15	" " "	5†	14	—	32.5	47.5	5.0	2.5	7.5	2.5	2.5	—	—	—
III_7	"	30	" " "	5†	12	2.8	13.9	36.1	22.2	13.9	—	—	11.1	—	—	—
III_7	"	30	" " "	5†	14	—	3.4	17.2	37.9	10.4	10.4	6.9	—	3.4	3.4	6.9
II_7	"	10	" " "	5‡	12	9.1	35.6	37.4	14.1	1.1	1.1	1.1	—	—	—	0.5
II_7	"	10	" " "	5‡	14	8.4	16.8	47.0	21.6	3.6	—	1.2	1.2	—	—	—
III_6§	"	10	" " "	5†	12	0.8	23.7	38.1	17.9	6.8	6.0	1.7	0.8	2.5	—	0.8
III_6§	"	10	" " "	5†	14	2.3	25.5	38.9	15.0	5.5	4.0	5.5	1.6	0.8	—	0.8
II_2	Lactalbumin.	10	Fat-soluble.	5†	12	9.4	40.5	36.5	12.2	1.4	—					
II_2	"	10	"	5†	14	—	20.3	53.1	17.2	7.8	1.6					
IV_5	"	15	"	5†	12	9.9	43.2	36.0	10.8	—	—					
IV_5	"	15	"	5†	14	3.8	22.6	47.2	22.8	—	1.9					
II_{2a}	"	30	"	5†	12	27.3	34.5	21.8	12.7	1.8	1.8					
II_{2a}	"	30	"	5†	14	36.0	12.0	18.0	20.6	6.0	2.0					

II_3	Lactalbumin.	10	Water-soluble.	None.	12	1.5	12.1	34.8	24.2	15.1	9.1	—	1.5	—	—	1.5	
II_3	"	10	"	"	14	3.3	13.1	18.0	41.0	8.2	3.3	8.2	3.3	1.6	—	—	
IV_8	"	15	"	"	12	3.1	34.6	22.3	23.8	13.1	0.8	2.3	—	—	—	—	
IV_8	"	15	"	"	14	8.3	28.8	26.7	24.2	6.7	5.3	—	—	—	—	—	
VI_1	"	30	"	"	12	3.0	19.9	28.8	38.0	7.6	3.0	—	—	—	—	—	
VI_1	"	30	"	"	14	8.3	1.7	30.0	35.0	16.6	5.0	3.3	—	—	—	—	
II_4	Lactalbumin.	10	None.	5‡	12	17.0	56.2	19.5	7.3	—							
II_4	"	10	"	5‡	14	16.6	39.0	27.8	16.6	—							
IV_6	"	15	"	5‡	12	5.1	50.0	31.6	12.5	0.7							
IV_6	"	15	"	5‡	14	10.3	20.6	41.1	16.9	1.8							
VI_2	"	30	"	5‡	12	11.9	45.2	23.8	19.0	—							
VI_2	"	30	"	5‡	14	23.6	20.6	38.2	15.0	2.9							
IV_{10}	Beef and oat.	17.7	Water- and fat-soluble.	5†	12	4.6	18.9	24.5	26.5	11.8	6.6	2.5	2.5	—	2.0	—	
IV_{10}	" "	17.7	" " "	5†	14	4.3	16.9	21.8	22.5	14.1	7.6	4.9	0.5	2.1	2.1	3.2	
II_1	Lactalbumin.	10	" " "	5†	12	4.2	29.8	34.0	8.5	6.4	12.8	—	2.1	—	2.1	—	
II_1	"	10	" " "	5†	14	—	14.6	34.1	29.5	7.3	2.4	—	4.9	2.4	4.9	—	
V_1	Lactalbumin, corn gluten. Ratio 1:1.	10	Water- and fat-soluble.	5†	14	2.4	23.8	42.8	21.4	4.8	1.2	2.4	—	1.2			
III_4	Corn gluten, cystine.	10	Water- and fat-soluble.	5†	12	12.2	49.0	30.0	2.8	2.8	3.0						
III_4	" " "	10	" " "	5†	14	10.5	41.1	32.6	10.5	3.2	2.1						
III_5	Corn gluten.	10	Water- and fat-soluble.	5†	12	13.6	41.9	40.7	3.7	—							
III_5	" "	10	" " "	5†	14	5.6	45.0	39.4	8.4	1.4							

* See Text-fig. 1, p. 333.

† Butter fat.

‡ Lard.

§ Same as II_1 except dextrin used for starch.

of fat that were present. Groups II_1, IV_4, III_7, and II_7 were fed lactalbumin in amounts of 10, 15, 30, and 10 per cent respectively with both vitamines and 5 per cent of fat. The results indicate that the amount of protein had no influence on the rate of development. When the water-soluble B accessory was absent and the percentage of lactalbumin varied from 10 to 30 per cent (Groups II_2, IV_5, and II_{2a}), the 30 per cent protein diet (II_{2a}) was somewhat the best. On the other hand, with no fat-soluble A and no fat in the diets (Groups II_3, IV_8, and VI_1), the results were slightly in favor of the 10 per cent protein diet (II_3). The non-vitamine groups (II_4, IV_6, and VI_2) show that the 10 per cent protein plane (II_4) was too low, while there was no difference between the 15 and 30 per cent diets.

To sum up, it would seem that in the main the amount of protein, whether 10, 15, or 30 per cent of lactalbumin, had comparatively little to do with the rate of development of the hind legs. There were some variations within the classes of vitamines that showed differences, but taken as a whole, one is hardly justified in placing too much emphasis upon these differences when the data for the corresponding periods are compared. This finding compares with rats where 10 per cent lactalbumin produces normal growth when properly supplemented.

If Group IV_{10}, fed the beef-oat protein diet, is compared with Group II_1, the percentage distribution is found to be in favor of IV_{10} when Period 14 is considered, but here again we are of the opinion that on the average it would be fairer to assume perhaps that there was not much difference, although when Fig. 8 is borne in mind we are almost forced to conclude that Ration IV_{10} was superior to Ration II_1. Here again it would seem that the amount of protein had little to do with the differences when we compare the data with that for Diet III_7. Furthermore, it does not seem to be a matter of vitamines or fat, for these are present in each diet. The beef or oats seemed to have carried other accessories or else the quality of protein was better adapted to the tadpole.

That lactalbumin was at least a fairly good protein for growth and development is shown from the results obtained by comparing it with a poor protein like corn gluten, which does not produce growth in rats (1, 2). Group III_5 was fed the same diet

as II_1 except that corn gluten was substituted for lactalbumin. There was a distinct difference between the two in favor of the lactalbumin. When corn gluten was supplemented with an equal amount of lactalbumin protein (Ration V_1), it is seen that the lactalbumin was able to stimulate development to some extent but not as much as when it was used as the sole protein.

Osborne and Mendel (1) found that cystine was able to supplement a low (9 per cent) casein diet and produce the same rate of growth as a 15 per cent casein ration, without cystine. In adding cystine to a 10 per cent corn gluten diet (Group III_4), we obtained slightly better results than when no cystine was present.

From the discussion on the amount and quality of protein in the diet, it would appear that the amount of protein, from 10 to 30 per cent, had little or no influence upon the development of the tadpole, but that the quality of protein was an important factor.

(*D*) *Influence of Kind of Carbohydrate.*—In Table IV, it is possible to make a comparison of the value of dextrin and starch, Groups III_6 and II_1 respectively. It is seen that there was no difference between the two.

CONCLUSIONS.

From the data herein reported upon the size and development of the frog larvæ (*Rana pipiens*), the following tentative conclusions are made, subject to revision in the light of studies which are being started for this season, and which will be carried out with a special effort to control more carefully the matter of temperature of water and the possibilities of dominance of one individual over the other.

1. A large amount of fat in the diet was very injurious to both growth (body size) and development of hind legs. Apparently the lower the percentage of fat the better the tadpoles thrived.

2. Vitamines—the water-soluble B and fat-soluble A types—appeared to be necessary for normal growth and development. The lack of the water-soluble B type was possibly more apparent than that of the fat-soluble A. When no vitamines were present, growth and development were distinctly retarded.

3. The amount of protein incorporated seemed to play a minor part in the development of hind legs when the per cent of lactalbumin ranged from 10 to 30—whether both "vitamines," one, or none, were present. On the other hand, the high protein fed tadpoles were largest (size) when both vitamines were in the diet.

4. The quality of protein was a factor which showed clearly that it should be borne in mind. Thus, lactalbumin, and beef and oats protein when incorporated in an otherwise complete ration brought about a good rate of development, while corn gluten protein gave poor results.

5 When dextrin was substituted for starch it had no effect either on growth or development.

6. The results taken as a whole show definitely that it is possible to adjust the diet so as to alter the size of the tadpole and the rate of metamorphosis, in respect to variations in such nutrients as fat, kind of protein, and vitamines. It should be borne in mind that other factors play an important part, such as temperature and food control. Too low temperatures tend to make the tadpoles sluggish, and too high temperatures produce abnormal changes which may result in death. The tendency to consume their dead makes it difficult at times to adjust the diet absolutely.

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EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. Photographic reproductions of tadpoles, showing the relative size as influenced by a high (I_1) and a low (II_1) fat diet.

FIG. 2. The diets given were both on the low fat plane. Diet II_1 contained the water-soluble B and the fat-soluble A vitamines, while Diet II_2 was lacking in the water-soluble type.

PLATE 3.

FIG. 3. Both diets contained the low percentage of fat. Diet II_1 was considered to be complete for normal growth and Diet II_3 was deficient in the fat-soluble A vitamine.

FIG. 4. Diet II_1 was compared with Diet II_4. The latter was lacking in both the water-soluble B and the fat-soluble A vitamines. These diets contained 10 per cent protein.

PLATE 4.

FIG. 5. The diets fed in this case were: complete (II_{1a}), and deficient in the vitamines (VI_2). They contained 30 per cent protein instead of 10 per cent as shown in Plate 3, Fig. 4.

FIG. 6. Both diets were complete. Diet II_1 had 10 per cent protein and Diet II_{1a}, 30 per cent protein.

PLATE 5.

FIG. 7. The two diets differed in the kind of carbohydrate used. Diet II_1 contained starch and Diet III_6 an equivalent quantity of dextrin.

FIG. 8. Diet IV_{10} differed from Diet II_1 in having the protein derived from desiccated beef tissue and rolled oats which had been extracted with ether. The former diet contained 17.7 per cent protein and the latter 10 per cent protein. These photographs should also be compared with those in Plate 4, Fig. 6, where Diet II_{1a} contained 30 per cent protein.

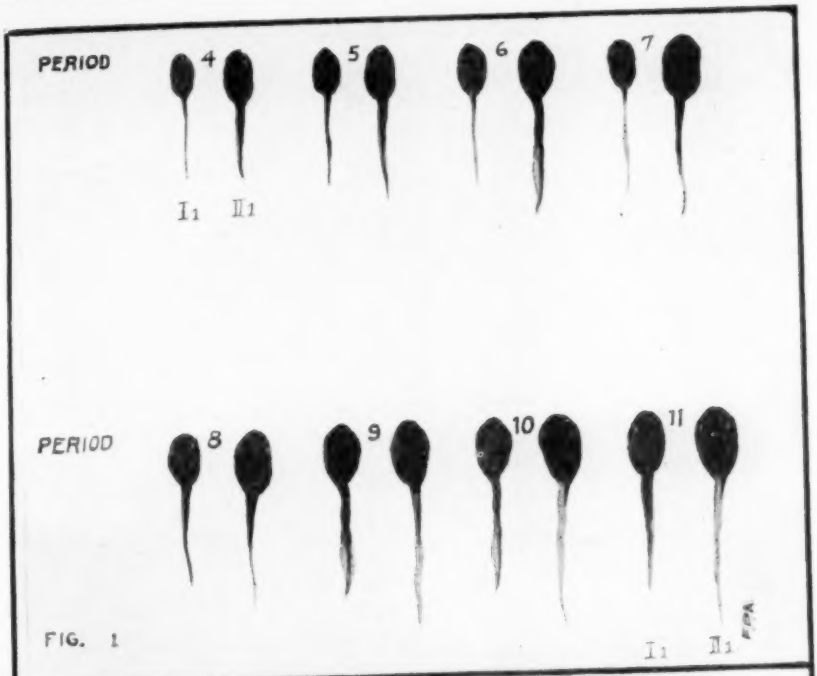

FIG. 1

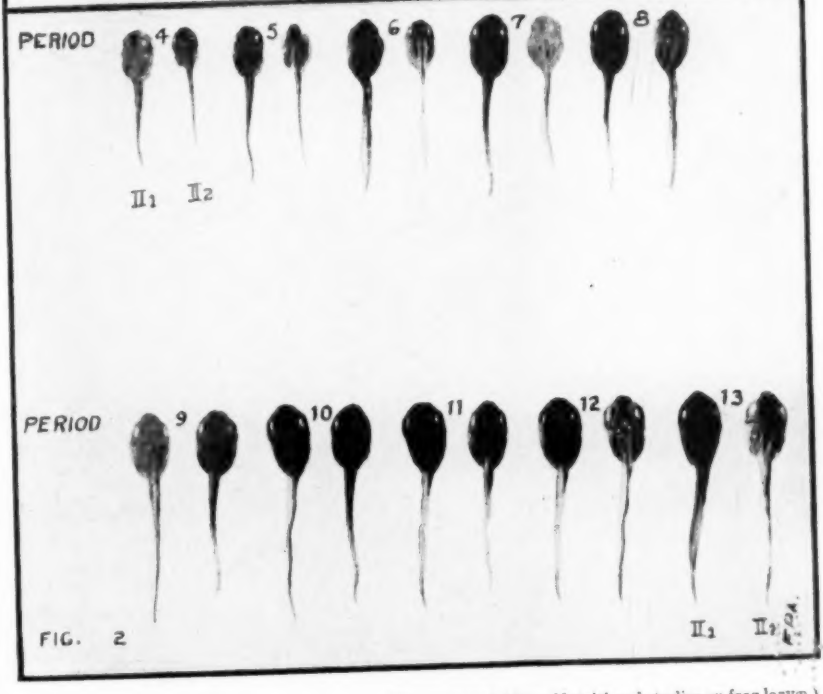

FIG. 2

(Emmett and Allen: Nutritional studies on frog larvæ.)

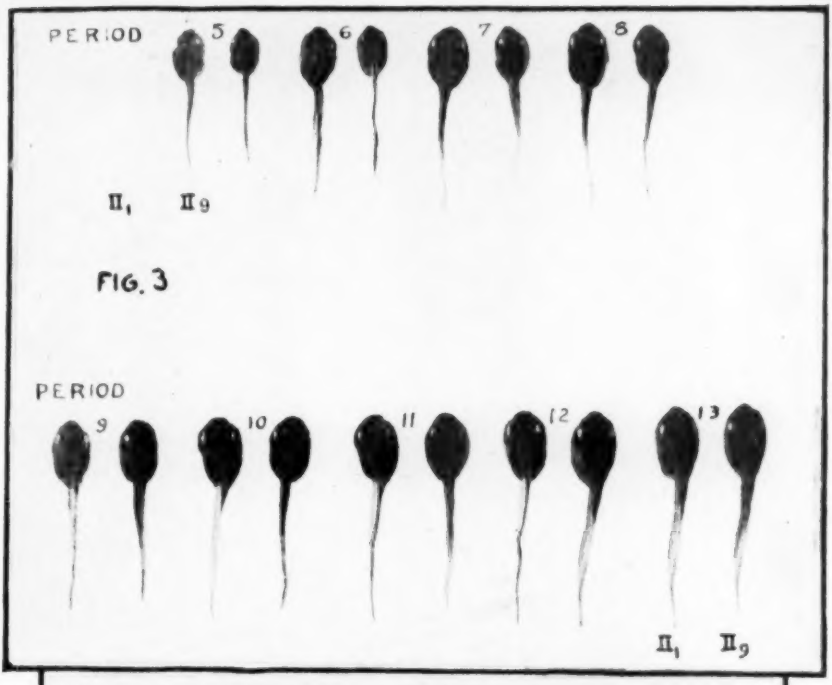

FIG. 3

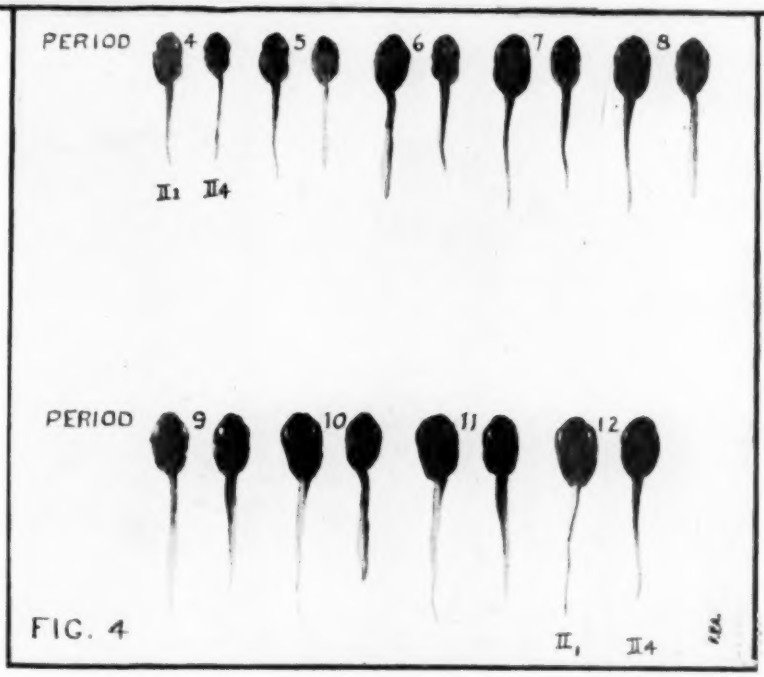

FIG. 4

(Emmett and Allen: Nutritional studies on frog larvæ.)

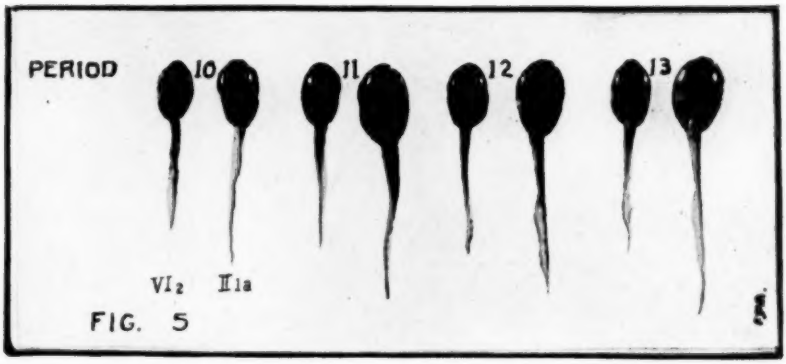

FIG. 5

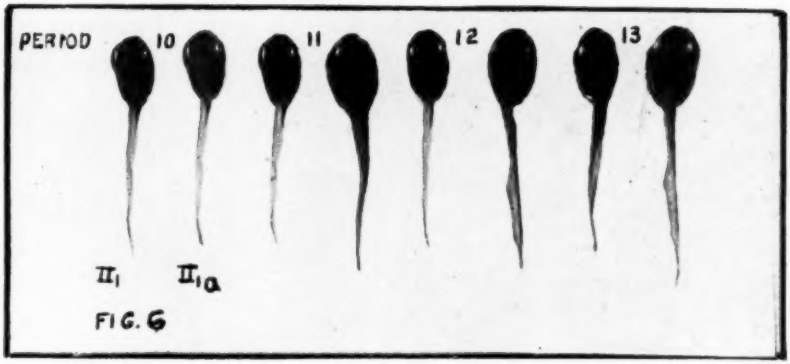

FIG. 6

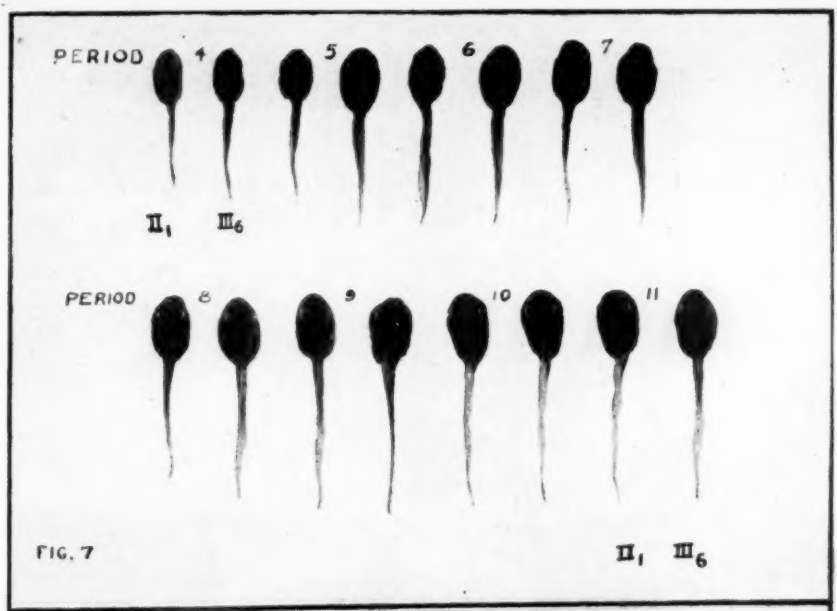

FIG. 7

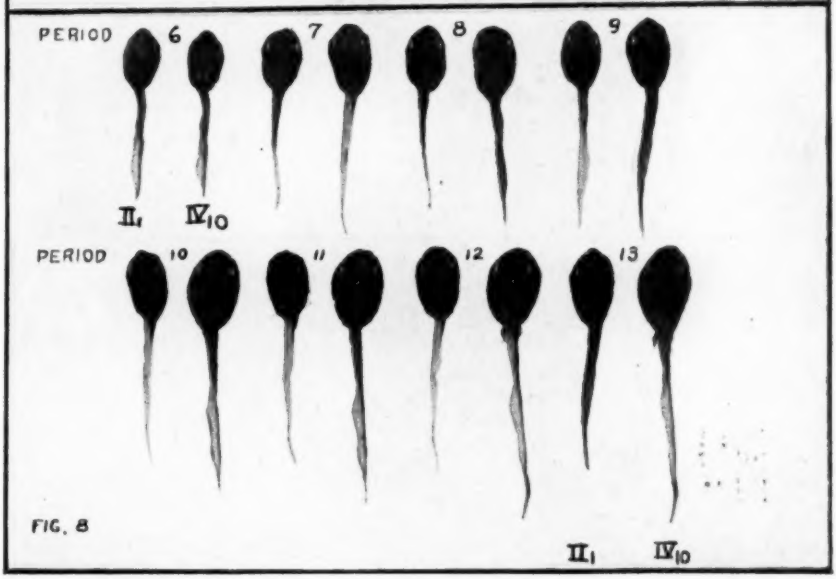

FIG. 8

(Emmett and Allen: Nutritional studies on frog larvæ.)

THE AGE AT WHICH TRYPSINOGEN APPEARS IN THE FETAL PANCREAS.

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INTRODUCTION.

The value of an investigation of the passive physiology of the embryo mammal is apparent to workers in many scientific fields. The physicochemical relationship which the factors concerned in embryonic growth bear to the growth and nutrition of the adult has been brought to light by the work of Mendel and his collaborators, Mitchell (1), Saiki (2), and Leavenworth (3). In this work the demonstration of the presence or absence of many vital products, including certain enzymes, in pig embryos of various ages, played an important part. Buxton and Shaffer (4) investigated the occurrence of certain enzymes in very young pig embryos, with special reference to a purely pathological problem. Their goal was the disproval of the theory of the fetal origin of certain mammalian neoplasms by contrasting the enzymes found in these growths with those found in the embryos. Jones and Austrian (5), working toward a closer differentiation of the nucleoprotein-digesting ferments, used the liver of the pig embryo of various ages to demonstrate the separate appearance of the individual ferments.

The fixation of a comparatively definite point in the growth of an animal, below which no appreciable amount of a proteolytic zymogen can be demonstrated in the pancreas, will lay the foundation for a large amount of work, anatomical, chemical, and physiological, and will give us a more complete understanding of the nature of this subject. It has been the sole object of this work to establish the time when the presence of a proteolytic zymogen can first be demonstrated in the pig fetus.

A review of the literature reveals no specific work with the embryonic pancreas having for its objective the determination of its proteolytic power, but the work of the men above mentioned offers some clue as to the time of onset of some of the special glandular functions. Buxton and Shaffer (4) found amylase and lipase present in extracts of whole pig embryos of 10 mm. size, oxidases at 26 mm., and proteolytic enzymes appeared to be variable, occurring between 26 mm. and 85 mm. Jones and Austrian (5) could first demonstrate adenase in the liver of fetal pigs of 150 mm. size, but guanase not until after birth. Catalases were shown to be present in the liver, lung, muscle, and brain as early as 65 mm., by Mendel and Leavenworth (3); aldehydase, as early as 80 mm. was found by Jacoby (6) in whole embryo preparations; while Batelli and Stern (7) in similar preparations found lipase in very young animals. Mendel and Mitchell (1) succeeded in obtaining lactase and maltase from extracts of intestinal mucosa of fetuses as young as 75 mm.

In the instances quoted above, the fetus of the domestic pig was employed, for the same reasons, presumably, as in the present instance; namely (1) the easy access to many litters of all ages at the slaughter houses, and (2) their larger size as compared with other accessible fetuses, enabling the dissection of the pancreas with facility. In all succeeding statements reference to the fetuses will be made in terms of millimeters, which represent, in the animals handled by myself, the linear distance from the base of the tail to the crown of the head when the animal is lying in its natural curvature on a flat surface. With reference to other work, although the method of measurement was not included in the articles, we may assume that approximately the same system was employed. Since from 4 to 12 fetuses were dissected in the litters employed, it is to be expected that in the larger animals (those over 100 mm.), a variation of a few mm. in the fetuses of the same litter was frequently found, and the average of the lengths was therefore taken for the record. Such a variation in individuals from a litter whose average length was less than 100 mm. caused me to suspect that the irregular animals were abnormal and they were therefore rejected for the preparation of extract.

Although some of the above results are at variance, a striking feature of a general survey is that the special glandular functions, particularly in the organs arising in connection with the intestinal tract, come into a measurable degree of development between 65 and 85 mm. The result of the work herein given serves to strengthen this generalization, and likewise harmonizes with the work of Corner (8) on the anatomy of the fetal pig's pancreas in various stages of development. He found that the practically homogenous plexus constituted by the anastomosing embryonic ducts, gives way to a coordinated tree-like pattern, constituted by ducts of various orders, the process being completed between the ages of 60 and 85 mm. He likewise found that the differentiation of the glandular units is begun about the same period. It is consequently interesting that the production of intracellular zymogen by the cells of this organ is coincident with the development of a mechanism for the output of these products.

EXPERIMENTAL.

It is unnecessary to describe the evolution of the method as described below, except for the statement in passing that the technique employed by Buxton and Shaffer (4) in their work on proteolytic enzymes, was found to be inadequate for any constant quantitative or even qualitative estimate of the digestive action of a pancreatic extract, and was therefore abandoned after several trials. In brief the criterion of this method was the appearance of a clear ring about a drop of a proteolytic solution on a milk-agar Petri plate, indicating the digestion of the casein in the neighborhood of the ferment.

In the adoption of a technique three factors had to be considered: (1) obtaining a potent extract of the pancreatic tissue in a utilizable amount from a series of animals of successive ages; (2) the exclusion of all factors tending to produce any effect on the final readings which would mask the results obtained from the action of the extract; and (3) the accurate measurement of the digestive action of this extract.

To insure the first and second conditions, the following technique was employed. The pancreas was dissected out of each fetus of the individual litters, taking care in the procedure to

prevent the bursting of the stomach or the inclusion of an appreciable amount of blood. All the glands from the same litter were then placed in stoppered bottles and allowed to stand at room temperature for 24 hours. This lapse of time served to encourage bacterial growth whose action, according to Mellanby and Wooley (9), transforms a maximum amount of trypsinogen into trypsin within this period of time. The bacteria which are supposed to bring about this activation are *Bacillus subtilis*, *Bacillus coli*, and Moeller's dung bacillus which are commonly present in the circulating air. In contrast to this theory, according to Morse (10) and Bradley (11), zymogens will be converted into enzymes in sterile surroundings, and to support this theory Nelson and Long (12) prepared proteolytic pancreatic extracts from freshly triturated gland, claiming that the extraneous tissue included contained sufficient enterokinase to activate a maximum amount of the extract. For the purpose of this work the interval of 24 hours in time gave a maximum amount of proteolytic enzyme.

The amount of pancreatic connnective tissue in ratio to the actual parenchyma, when collected in the entire gland mass, is one of the variable factors tending to make the proportional amount of functional enzyme appear less in the tabulated results of the younger embryos than really exists. The second disturbing factor which is, however, negligible for the series of litters done simultaneously, but which causes a variation of some magnitude in the results of series done on different days, is the unknown degree to which activation has proceeded. Because of this latter factor the results of the experiments have been placed in three separate columns, as noted in Table I, each column consisting of the series of litters employed simultaneously.

After the activation of the zymogen has taken place a glycerol extract of the gland was prepared in slightly basic medium, by triturating it with washed quartz sand in the proportion of 1 gm. of gland to 5 cc. of glycerol and 5 cc. of 0.5 N Na_2CO_3. Vernon (13) has shown that glycerol pancreatic extracts are more stable than alcoholic extracts while no other extractive is as effective as either of these two.

The trituration was continued until a smooth milk-like suspension of the gland in the fluid was obtained and 0.2 cc. of

this freshly prepared material was pipetted into each of four clean dry glass tubes of about 5 cm. length and 4 mm. inside diameter. To each tube was then added 2 cc. of 0.5 N Na_2CO_3, as this amount furnished approximately the concentration of OH-ions, found by Vernon (13) to give the optimum reaction for the tryptic digestion of protein. This optimum alkalinity was found by Vernon (13) to be about $\frac{1}{1,200}$ N OH^- and these results were also approximately obtained by Michaelis and Davidsohn (14) and later by Long and Hull (15), while the explanation for the necessity of this optimum alkalinity was given by Robertson and Schmidt (16).

As a relatively constant and easily accessible source of water-soluble, digestible protein, minced coagulated egg white, which had been soaked for 24 hours and then partially desiccated, was added in 0.2 gm. masses to the tubes to serve as the substrate for the ferment. To prevent the action of proteolytic bacteria, it was necessary to employ a bactericide. The choice of a proper antiseptic was difficult in that it must not be toxic to the ferment like the phenols or the salts of the heavy metals (mercury, copper, arsenic, or silver), nor a protein coagulent like the latter mentioned salts, nor must it be volatile like chloroform or toluene, since the tubes are to be sealed and heated. The cyanides and fluorides are the salts approximating the necessary conditions most closely, although both are markedly toxic. However, the fluorides are the less toxic of the two and Vernon (13) has shown that NaF is less so than NH_4F, a concentration of 0.5 per cent NaF sufficing to inhibit bacterial growth completely without materially altering the speed of the protein hydrolysis. On the basis of this work I added 0.26 cc. of 4 per cent NaF to each of the tubes containing the extract and protein, to obtain the optimum concentration.

Holding the tubes at a level above the contained fluid in order to insure that the material would remain cool, the open ends were drawn out and sealed in a flame to guard against the possible factor of evaporation occurring irregularly during the processes of sealing.

Of the four identical tubes, two were heated in the water bath to a temperature of 97–100°C. for a period of 5 minutes, which procedure has been found by Ohta (17), de Souza (18), and

many other investigators to destroy completely the potency of trypsin. We now have four tubes apparently identical as to their contents except that two contain an active ferment, if such existed in the original pancreas, while the other two tubes are entirely inactive. They were now incubated at 38°C. for 24 hours, this being approximately the optimum temperature and time for tryptic hydrolysis of protein. After incubation the tubes were centrifuged until the supernatant fluid was freed from all suspended matter. If ferment had been present in the unheated tubes, the product of its hydrolysis would now be included in the fluid contents of the tubes.

The third condition, as mentioned in the beginning of this section, namely, the accurate mensuration of the digestive action of the pancreatic extract, must now be fulfilled. This could be accomplished through the measurement of the amount of protein digested in the tubes containing the enzyme, and several methods were available. The biuret test and Kjeldahl estimation of nitrogen have been employed by Vernon (13), the polariscopic method by Koelker (19), the colorimetric method by Harding and MacLean (20), and the Van Slyke method by Long and other investigators (21). Owing to the extremely small amount of utilizable fluid, I adopted, at the suggestion of Professor Robertson (22), the refractometric method as first developed by Reiss (23) for determining the total protein in blood serum. and later perfected by Robertson (22) for the determination of the individual blood proteins.

The clear supernatant fluid was drawn off from the tubes, after breaking them open near the top, and the refractive indices of each specimen determined in a Pulfrich refractometer. The readings are made in angles of total reflection and converted into indices of refraction by a table furnished with the instrument. The difference between the refractive index of the inactivated tube, serving as a control, and the tube containing the protein split-products represents the amount of egg white digested. To minimize the experimental error each series was done in duplicate using two control tubes and two active tubes, and as shown by the tabulated results, the duplicates agree to ± 0.00008, which corresponds to an error of 1 second in reading.

Table I gives the complete results of this series of embryos and demonstrates a lack of a measurable amount of proteolytic

TABLE I.

Refractometric Determination of the Relative Amount of Protein Digested by Pancreatic Extracts from Pig Embryos of Various Ages, by Comparison of Differences of the Indices of Refraction in Active and Control Tubes (in Duplicate).

Size.	Approximate age. (After Coe, 24.)	Indices of refraction.		Refractivity due to the solution of split products. (Average of two determinations.)		
		(1)	(2)	Series 1.*	Series 2.†	Series 3.‡
mm.	*days*					
	Adult.	A- 1.34148			0.00088	
		C- 1.34059				
220	89	A- 1.33959		0.00072		
		C- 1.33887				
133	70	A- 1.34098			0.00074	
		C- 1.34024				
123	67	A- 1.34008	1.34008		0.00061	
		C- 1.33943	1.33951			
105	63	A- 1.33855	1.33855	0.00048		
		C- 1.33807	1.33807			
100	62	A- 1.34131	1.34131			0.00086
		C- 1.34049	1.34041			
95	61	A- 1.34016	1.34024		0.00065	
		C- 1.33959	1.33951			
92	60	A- 1.34065	1.34073			0.00094
		C- 1.33975	1.33975			
88	58	A- 1.34000	1.33992			0.00041
		C- 1.33959	1.33951			
78	55	A- 1.34163	1.34163		0.00016	
		C- 1.34147	1.34147			
78	55	A- 1.34081	1.34000			0.00024
		C- 1.34065	1.33967			
75	53	A- 1.34041	1.34041			0.00021
		C- 1.34016	1.34024			
72	52	A- 1.33727	1.33719	−0.00004		
		C- 1.33727	1.33727	0.00000		
65	50	A- 1.33975	1.33967			−0.00004
		C- 1.33975	1.33975			0.00000

A = Tubes containing active enzyme. C = Control tubes.

* Series started on May 25, 1918.

† Series started on May 30, 1918.

‡ Series started on June 4, 1918.

It is to be noted that the actual variance between the duplicate pairs (1), (2) always comes within the limit of the error of the apparatus (± 0.00008), for each determination. Results lying between 0 and ± 0.00008 have been taken as 0 since this is wholly within the magnitude of the experimental error.

type of zymogen in fetuses up to the age of 75 mm. From this age up to 92 mm. a rapid increase in the zymogen content is noted which then becomes practically constant for a unit quantity of gland. This increase in zymogen content is more clearly

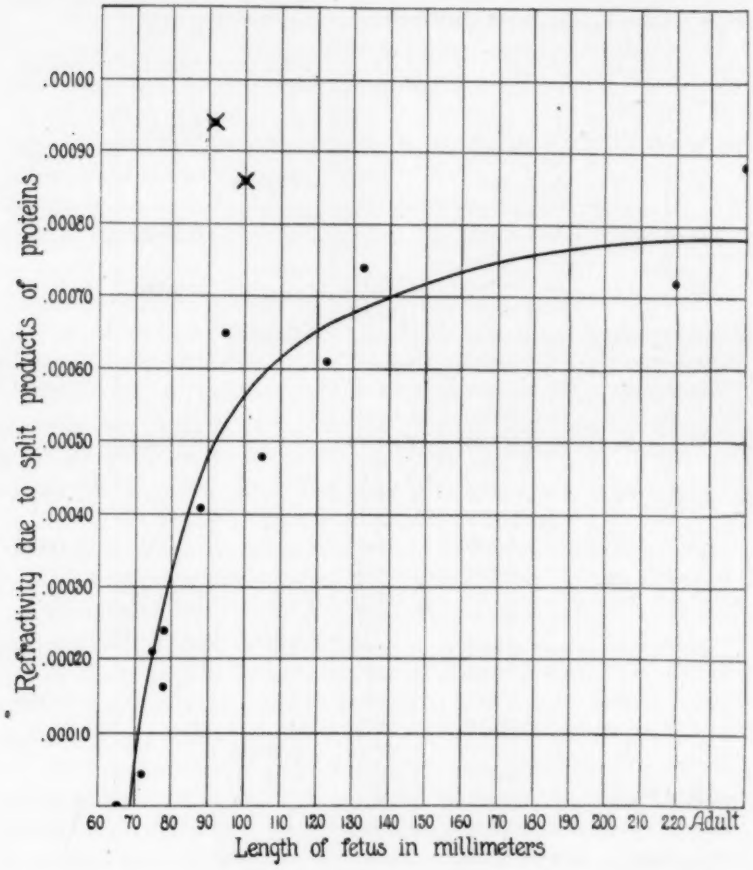

FIG. 1.

demonstrated in the curve approximating an average of the values plotted graphically (Fig. 1). The high digestive power of two tubes of the same series, which throws them out of the zone traversed by the curve, may be explained by certain factors

which would be constant for an individual series of a particular day. Since at best, an age-length ratio is only a rough estimate, the ages herein quoted as based on an unofficial statement of Coe (24), should be considered only as serving to give a general impression of the time prior to birth at which this alteration in zymogen content, as discussed above, occurs.

CONCLUSIONS.

1. Between the lengths of 65 and 72 mm. or the approximate intrauterine ages of 50 and 53 days of the pig embryos, the pancreas develops an amount of trypsinogen, which, when activated, is sufficient to digest enough protein to be measured by the refractometric method.

2. At a length of 95 mm. or an intrauterine age of from approximately 60 to 62 days, a maximum is reached in the trypsinogen content.

3. Although the two facts may not be related, the age of onset of a measurable amount of trypsinogen in the pancreas agrees closely with the time at which Corner has shown that a dendritic duct system is substituted for the primitive anastomotic tubules.

Since this study has shown that by the methods employed the accumulation of recognizable zymogen in the pancreas has a rather definite point of beginning and reaches a definite maximum, it becomes increasingly interesting to see whether finer structural details in the pancreas are coincident with this. It will be recalled that Bensley (25) and other workers claim to have recognized cytological criteria for enzyme-secreting cells. These morphological aspects of the question will constitute a later communication by Dr. Corner.

I am indebted to Dr. T. B. Robertson for his assistance in suggesting alterations toward perfecting the method of attack on this problem; to Dr. H. M. Evans for the original suggestion; and to Dr. C. L. A. Schmidt for his aid in revision of the manuscript.

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THE METABOLISM OF BILE ACIDS.

I. A QUANTITATIVE METHOD FOR ANALYSIS OF BILE ACIDS IN DOG'S BILE.

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An unusual opportunity for a comprehensive study of the bile acids presented itself to us in this laboratory because of the presence of a number of bile fistula dogs under *careful routine* observation. These bile fistula dogs were under observation for the study of bile pigments. They present normal factors of weight, activity, and appetite when under the laboratory routine care which has been described in detail by Hooper and Whipple (8).

In order to study satisfactorily the metabolism of the bile acids it is necessary to have a method for the analysis of bile acids which is relatively simple and accurate and which does not require large quantities of material for analysis. The method must be specific for bile acids and react negatively with the other substances in bile and it must not be obscured by any substances which may be present in bile fistula bile. We know of no published method which meets all these requirements.

As a preliminary step we undertook to test a number of the clinical methods for the determination of bile acids, some of which are known to be inaccurate and others of which are claimed to be merely qualitative. Without exception we have found these

*This series of papers on Bile Acid Metabolism was completed just prior to the death of Miss Foster from influenza pneumonia. The work should stand as a memorial to her enthusiasm, patience, and spirit of truthful research. This work was submitted as a thesis for her degree of Doctor of Philosophy, University of California.

relatively simple clinical methods to be grossly inaccurate and useless for analysis of bile salts. This statement applies to a recent method advocated by Hoover and Blankenhorn (9). No controls are given by these workers to show that many other substances present in abnormal sera may not have been responsible for the positive bile acid reaction noted in their experiments. For a discussion of these possibilities refer to the paragraph below on Pettenkofer's test.

Some of the more elaborate chemical methods for the extraction of bile salts from bile are relatively accurate, but the large quantity of material used and the amount of time required make frequent analyses at short intervals impracticable. These extraction methods are very expensive as well as time-consuming. The older methods may be divided into three groups: (1) methods in which the bile acids are separated and weighed; (2) methods in which the bile acids are calculated from the sulfur content in whole bile; and (3) methods which depend on the color reactions of cholic acid. There are possibilities of error in all these methods, as will be pointed out later.

Methods in Which the Bile Acids are Separated and Weighed.

Huppert's Method—1864.—Huppert (11) modified Neukomm's (17) method for bile salts in urine and applied it to the bile and blood in the following way. The albumin was first coagulated with alcohol and the precipitate carefully extracted with additional amounts of alcohol. The whole extract was evaporated, dissolved in water, freed from fat by ether extraction, neutralized, and $Ba(NO_3)_2$ added to separate the fatty acids, soaps, and any remaining protein. The washed precipitate was supposedly free from bile acids. The filtrate and wash water were precipitated with lead acetate and the precipitate washed with water to free from excess acetate. The precipitate was then washed out with alcohol, heated, and Na_2CO_3 added. After it was evaporated to dryness, the residue was extracted with absolute alcohol; the alcohol was evaporated to dryness; and the residue was dissolved in water. This water solution was filtered into a weighed dish and dried to constant weight.

Socoloff's Method—1875.—Socoloff (19) published a method in which the bile acids present were calculated by the amount of bile soluble in alcohol. This work was done in Hoppe-Seyler's laboratory using a method very much like the one described in the following paragraph.

Hoppe-Seyler's Method—1881.—Hoppe-Seyler's method (10) was used by Pfaff and Balch, Stadelmann, and others. The bile was dried to constant weight at 110°C. and the residue completely extracted with boiling

absolute alcohol. The extract was allowed to stand 24 hours, filtered, and evaporated to dryness at 110°C. The residue was dissolved in absolute alcohol, evaporated to a small volume, cooled, and precipitated with a large excess of absolute ether. The bile salts which crystallized out were dried at 110°C. and weighed. Stadelmann (21) evaporated the bile to a thick syrup and allowed it to stand in contact with alcohol on the water bath some time in order to coagulate the protein and give a clear filtrate. He then repeated the extraction three times with boiling 96 per cent alcohol. He dissolved the final precipitate in water and dried to constant weight.

Croftan's Method—1902.—Croftan (3, 4) estimated the bile acids by coagulating the albumin, evaporating the filtrate and washings, and precipitating with absolute alcohol to free from salts. The filtrate was diluted with water, precipitated with basic lead acetate and ammonia, and the precipitate extracted with absolute alcohol and filtered hot. This solution of bile salts was dried to constant weight. (According to Croftan the mucin holds with it a large amount of bile acid and they are so closely bound that it is impossible to separate them even by repeated washings.)

Goodman's Method—1906.—Goodman (5) hydrolyzed 50 gm. of bile with 125 gm. of 60 per cent KOH in a reflux condenser for 24 hours. The solution was extracted five times with 75 cc. of freshly distilled petroleum ether in order to extract the cholesterol. The last of the petroleum ether was evaporated off on a water bath and the solution precipitated with 5 per cent $BaCl_2$ to separate the higher fatty acids. The precipitate was extracted with boiling water and the filtrate and washings evaporated to 200 to 300 cc. The solution was cooled in ice and salt, and acidified with HCl. After standing 2 hours the cholic acid which crystallized out was filtered off, washed, dried, and extracted in a Soxhlet apparatus with acetone. In about 5 hours all the cholic acid was dissolved and the solution was evaporated and dried to constant weight.

Methods in Which the Bile Acids Are Calculated from the Sulfur Content in Whole Bile.

Spiro's Method—1880.—The sulfur method as used by Spiro (20) consisted in fusing a definite amount of bile (usually 50 cc.) in a silver dish with KOH and KNO_3. The mixture was dissolved in water, supersaturated with HCl, and precipitated hot with $BaCl_2$. This precipitate was filtered on a paper of known ash, washed well, and dried. The main part of the precipitate was transferred to a porcelain crucible. The paper with the remainder of the precipitate was ignited and weighed. The main part of the precipitate was weighed, a few drops of concentrated H_2SO_4 added, washed out well with water into another filter paper, and this was weighed. The difference in weight showed the amount of the impurity. From this difference was calculated the $BaSO_4$ in the other portion of the precipitate which was weighed with the ash of the larger filter paper, and the total amount of sulfur was calculated from this corrected value.

Von Bergman's Method—1904.—Von Bergman (2) precipitated a 24 hour collection of bile with many volumes of 96 per cent alcohol. The mucin precipitate was filtered off and washed six or eight times with alcohol. The combined washings and filtrate were evaporated to a definite volume and an aliquot part used for the determination of the combined sulfur.

Methods Based on the Color Reactions of Cholic Acid.

Pettenkofer's Method.—This test as outlined in Hammarsten (6) is performed by dissolving a small amount of bile in concentrated sulfuric acid and warming to 60 or 70°C. A 10 per cent solution of cane sugar is added drop by drop. A beautiful red color develops which turns bluish violet on standing. The red liquid shows a spectrum with two absorption bands, one at F and one between D and E. The test fails if the solution is heated too hot, or if too much sugar is added (on account of the sugar carbonizing). Also, if impurities are present in the acid, such as H_2SO_3 or the lower oxides of nitrogen, the reaction fails. Proteins, amyl alcohol, oleic acid, morphine, etc., give a similar color so that it is necessary to carry out the spectroscopic examination also. Ville and Derrien (23) state that vanillin and anisaldehyde give the same color, and cholesterol gives a similar color. This method has been improved upon by Mylius and von Udránszky, who advise the use of a 1 per cent solution of furfurol in place of the sugar. Von Udránszky (22) emphasizes the fact that pure bile acids are necessary and suggests decolorizing the bile with charcoal and using an alcoholic solution of the residue. To each cc. of alcoholic solution, add one drop of furfurol and 1 cc. of concentrated sulfuric acid, and warm gently, This will detect $\frac{1}{20}$ to $\frac{1}{3}$ mg. of cholic acid. According to Hammarsten, the protein and fat should also be removed by neutralizing the bile and adding alcohol to at least 85 volumes per cent pure alcohol. The solution is filtered and the protein extracted with fresh alcohol, and the alcoholic extract evaporated to dryness. This residue is extracted with absolute alcohol, filtered, and the extract evaporated to dryness. The residue is extracted with ether, dissolved in water, and the solution precipitated with basic lead acetate and NH_4OH. The precipitate is washed and dissolved in boiling alcohol, filtered, and made alkaline with a few drops of NaOH. This solution is evaporated to dryness, the residue extracted with absolute alcohol, filtered, and precipitated with ether. This solution may be used for Pettenkofer's test, but even this may contain phosphatides and they give the same color reaction as do the bile acids.

Inouye and Ito (12) reported that when vanillin and concentrated sulfuric acid are added to solutions of bile acids a red line is formed at the line of contact. If the fluids are then mixed, the solution changes to a red-brown and then violet. When this solution is diluted with glacial acetic acid, an adsorption band is shown at B. This reaction is sensitive with taurocholic acid in a dilution of 1:11,000 and with cholic acid 1:22,000.

Jolles (13) published a method in which the bile acid (2 to 3 cc. of 1 per cent solution) is mixed with rhamnose (1 to 2 drops of 5 per cent solution)

and boiled with 2 to 3 cc. of concentrated HCl. A red color is produced followed by a green fluorescence which is due to the formation of methyl furfurol aldehyde. The reaction can be carried out by 0.005 to 0.0001 gm. of pure acid. It is not affected by urea, albumin, carbohydrates, hydrocarbons, or acids of the aliphatic or aromatic series, glycocoll, taurine, or cholesterol.

Authors' Method.

Dog's bile contains only taurocholic and taurocholeic acids, and on hydrolysis these split into taurine and cholic and choleic acids. Taurine is amino ethyl sulfonic acid, $CH_2NH_2CH_2HSO_3$. It acts like an α-amino-acid, and gives off its NH_2 quantitatively in 3 minutes in the Van Slyke amino nitrogen apparatus. This method consists in hydrolyzing a definite amount of bile with NaOH, thus splitting the bile acid, and then determining the amount of NH_2 in the taurine. Neither taurocholic nor taurocholeic acid gives off NH_2 before hydrolysis. In other animals both taurocholic and glycocholic acids are present, and it is therefore not certain that the method in its present form can be applied to the bile of animals other than the dog.

For the determination 5 cc. of bile cleared in the centrifuge and measured in a calibrated pipette are precipitated with 40 cc. of 95 per cent alcohol, and heated to the boiling point to insure a complete solution of the bile acid which might be held with the mucin precipitate. After cooling, the mixture is made up to 50 cc. in a cylinder with 95 per cent alcohol, and passed through a dry filter paper. Two specimens of 20 cc. each are evaporated to dryness. One is washed out with water and made up to 10 cc. in a calibrated flask. 2 cc. samples of this are used to determine the amount of NH_2 present before hydrolysis. The other is washed out quantitatively with 6 cc. of 8 per cent NaOH into a test-tube. The test-tube is loosely stoppered and placed in a boiling water bath for 5 hours. The contents are washed out into a 10 cc. calibrated flask and made up to volume with distilled water. 2 cc. samples of this are used to determine the amount of amino nitrogen due to the hydrolysis of the bile acids. The nitrogen is then figured on the basis of 1 cc. of bile for both specimens by multiplying the mg. of amino nitrogen found by 2.5 and subtracting the unhydrolyzed amino nitrogen from that due to hydrolysis. This figure is multiplied by the total volume of

bile for the 6 hour collection, which gives the total output of amino nitrogen for 6 hours. The bile acids are figured as taurocholic acid by multiplying by 36.72, the factor obtained by dividing the molecular weight of taurocholic acid by the atomic weight of nitrogen.

Quantitative Estimation of Taurine.

Table I shows that taurine gives up its NH_2 quantitatively with 3 minutes shaking in the Van Slyke amino nitrogen apparatus. The reaction is complete in that time even at as low a temperature as 13°C.

TABLE I.

Quantitative Estimation of Taurine.

Taurine.	Nitrogen gas.	Temperature.	Pressure.	Correction.	NH_2—N found.	NH_2—N theoretical.
mg.	*cc.*	*°C.*	*mm.*	*cc.*	*mg.*	*mg.*
46.00	9.11 9.10	18	760	0.10	5.157	5.152
9.785	2.06 2.07	22	756	0.10	1.095	1.096
10.58	2.17 2.17	13	759	0.14	1.189	1.185

Analysis of Sodium Taurocholate.

To test out the method a specimen of sodium taurocholate was analyzed for N, S, and ash, and the NH_2 yielded after hydrolysis. Table II shows that the content of both N and S of the sodium taurocholate used was about 85 per cent of their theoretical values. The 13.3 per cent ash partly accounts for the low values of N and S. Sodium taurocholate should have had only 4.3 per cent sodium in the ash if one hydrogen atom was replaced by Na.

The solutions used in Table III were hydrolyzed 5 hours in a boiling water bath and made up to 10 cc. in a calibrated flask. NH_2 determinations gave the following results.

Number.	Amount used.	Nitrogen gas.	Temperature.	Pressure.	Correction.	Amino nitrogen in 10 cc. solution.	Total NH_2—N in 10 cc.	Hydrolysis $\frac{NH_2}{N}$.
	cc.	*cc.*	*°C.*	*mm.*	*cc.*	*mg.*	*mg.*	*per cent*
1	1	1.83 1.82	20	758	0.10	9.74	10.29	94.7
2	1	1.28 1.26	20	758	0.10	6.62	6.86	96.5
3	2	1.31 1.31	20	758	0.10	3.42	3.43	99.8
4	2	0.33 0.33	20	758	0.10	0.65	0.68	95.6

TABLE II.

Analysis of Sodium Taurocholate.

Sodium taurocholate.	Total N.		Theoretical N.	Per cent of theoretical value.	Ash.
mg.	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
779.6	17.15	2.20	2.60	84.62	13.3

Sodium taurocholate.	$BaSO_4$	Total S.	Theoretical S.	Per cent of theoretical value.
mg.	*mg.*	*per cent*	*per cent*	*per cent*
250*	92.6	5.09	5.96	85.42

* The sulfur determinations were very kindly carried out by Dr. C. L. A. Schmidt.

TABLE III.

Hydrolysis of Sodium Taurocholate.

Number.	Sodium taurocholate solution.*		Water added.	Sodium taurocholate.	16 per cent of NaOH added.	NaOH in solution.
	cc.	*mg.*	*cc.*	*per cent*	*cc.*	*per cent*
1	3	467.7	0	15	3	8
2	2	311.8	1	10	3	8
3	1	155.9	2	5	3	8
4	3†	31.2	0	1	3	8

* 3.8980 gm. were made up to volume in a 25 cc. flask and definite amounts were used.

† Solution prepared by diluting 1 cc. of the original solution with 14 cc. of distilled water.

Table III shows that the hydrolysis of this specimen of sodium taurocholate in from 1 to 15 per cent solutions was approximately 100 per cent in 5 hours when the alkali present was 8 per cent during the hydrolysis.

This complete analysis was carried out on another specimen of sodium taurocholate with the same results.

Normal Constituents of Whole Bile.

According to Hoppe-Seyler (10) the normal constituents of whole bile are bile salts, bile pigments, cholesterol, mucin, ethereal sulfates, conjugated glucuronic acids, fats, soaps, a trace of urea, jercorin and other phosphatides, hydrochloric and phosphoric acids, and sulfuric acid as Na, P, Ca, Mg, Fe, and Cu salts. Marshall and Davis (16) found the same amount of urea in the bile as in the blood—32 mg. of urea per 100 cc. 1 cc. of bile would then contain 0.149 mg. of N, but Van Slyke states that only 3 per cent of N in the urea is given off in the first 3 minutes, *i.e.* 0.00447 mg. of N, and that amount is not sufficient to cause an appreciable error in this method. Traces of amino-acids have been found in disease, but these would give off their NH_2 nitrogen in the unhydrolyzed specimen, and thus would be corrected for. Stadelmann thinks that glycocholic acid may be present in dog's bile. The generally accepted opinion is that dog's bile contains no glycocholic acid or at the most a small trace. Even the presence of glycocholic acid sufficient to make up 10 per cent of the hypothetical bile acid mixture, would introduce no appreciable error, figuring all the bile acids as taurocholic acid. This is because of the large size of the cholic acid molecule and the similarity of weight of the molecules of glycocol and taurine. None of the other constituents set free NH_2 groups on hydrolysis except mucin. To obviate this error we precipitated the mucin with 10 volumes of 95 per cent alcohol. In one set it was filtered immediately; in a second, the alcohol was allowed to remain in contact with the mucin for 24 hours; and in a third, the alcoholic mixture was heated to its boiling point, cooled, and filtered immediately. Since Croftan showed that the mucin precipitate could not be freed of bile acid by extraction, we made the mixture to a definite volume with alcohol and then took an aliquot part of the filtrate. This would insure a uniform mixture of bile salts.

Table IV shows that mucin is best removed by alcohol heated to its boiling point. The NH_2 given off by the unhydrolyzed bile may be due to traces of urea or to some splitting of the bile acid. It is always proportional to the bile acid content, but not in exact ratio. The unhydrolyzed mucin-free bile gives a higher NH_2 in some cases than whole bile. This may be due to a slight splitting of the bile acid. The hydrolyzed whole bile gives off decidedly more NH_2 nitrogen than the mucin-free bile. This shows that the mucin is decomposed by hydrolysis into NH_2-

TABLE IV.

Whole Bile and Mucin-Free Bile.

Dog.	Unhydrolyzed bile.		Hydrolyzed bile.			
	Whole.	Mucin-free.	Whole.	Mucin-free.		
	NH_2–N* per cc. of bile.	NH_2–N per cc. of bile.	NH_2–N per cc. of bile.	NH_2–N per cc. of bile filtered immediately.	NH_2–N per cc. of bile after 24 hours.	NH_2–N per cc. of bile heated to boiling.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg*
17–151	0.194	0.245	1.210	0.924	1.012	0.915
16– 15	0.051	0.072	0.277	0.188	0.144	0.203
15– 22	0.205	0.245	1.386	1.185	1.214	1.276
18– 23	0.080	0.072	0.452	0.216	0.289	0.290

* The readings on the NH_2 apparatus have been omitted for simplicity.

containing substances (amino-acids). The mucin precipitated by heating the mixture of bile and alcohol to boiling seems to give the quickest and most satisfactory results.

Sodium Taurocholate Added to Bile.

To ascertain if the amino nitrogen determined in hydrolyzed bile is really specific for the bile acid, three specimens of the same bile were hydrolyzed after adding different known amounts of sodium taurocholate to each. A control was run on the bile and on the sodium taurocholate solution.

Table V shows that known amounts of sodium taurocholate added to bile can be recovered quantitatively with this method. The theoretical increase is figured from No. 1. The error is about 6 to 8 per cent loss. Since our conclusions are based on

decided increases in bile acid excretion in our experiments, a loss due to the method would have little significance in the analysis of results.

TABLE V.

Known Amounts of Sodium Taurocholate Added to Bile.

Number.	Bile.	Sodium taurocholate (15 per cent solution).	NH_2–N increase.	NH_2–N increase due to sodium taurocholate.	Theoretical increase.	Error (loss).
	cc.	*cc.*	*mg.**	*mg.*	*mg.*	*per cent*
1	0	1		1.150		
2	5	3	4.061	3.229	3.45	6.4
3	5	2	2.953	2.121	2.30	7.8
4	5	1	1.914	1.082	1.15	6.1
5	5	0	0.832			

* The readings for the NH_2 have been omitted for simplicity.

A Six Hour Collection of Bile.

Table VI shows the same total for three periods of 2 hours each as for one period of 6 hours. This speaks for a thorough mixture of the thick viscous bile and its contained bile acids and shows that a single estimation is accurate for the total excretion. It also gives confirmatory evidence of the accuracy of this method.

TABLE VI.

Mixed Collection of Bile.

Time.	NH_2–N per 1 cc. of bile.	Volume.	Total NH_2.	
			Output.	Per 6 hours.
hrs.	*mg.*	*cc.*	*mg.*	*mg.*
1–2	1.174	15	17.61	
3–4	0.553	14	7.74	
5–6	0.558	16	8.93	34.28 Total.
1–6	0.753	45	33.88	33.88 Total.

Variations in the Concentration of Alkali.

The amount and concentration of alkali used are of minor importance as can be seen in Table VII. The same specimen of bile was hydrolyzed with varying amounts and concentration of

NaOH and the results show that the bile acid was completely hydrolyzed in all the tubes. Table VII also shows that slight variations in the amount and concentration of alkali used for the hydrolysis do not affect the accuracy of the method.

TABLE VII.

Strength of NaOH Used.

Tube.	NaOH		NH_2–N
	cc.	*per cent*	*mg.*
1	5	8	0.364
2	6	8	0.364
3	7	8	0.350
4	6	6	0.350
5	6	12	0.364

DISCUSSION.

Hammarsten (6 and 7) found jecorin, lecithin, and other phosphatides in bile, and all contain nitrogen and sulfur. Since the nitrogen is in the choline radical, it does not interfere with the present method, as choline on hydrolysis with alkalies yields trimethylamine and glycol, and trimethylamine does not react with nitrous acid. But sulfur does interfere with the bile acid methods as determined by the sulfur content.

The phosphatides cause a decided error in the methods in which the bile acids are weighed. Long and Gephart (15) have found it impossible to separate bile acids from lecithin even with acetone. Bile salts can hold in stable solution 80 per cent of their weight of egg lecithin. Part of this can be separated by precipitation, but the amount remaining with the bile acids and not separated by acetone is much in excess of that contained in any bile. Ethereal sulfates are present in some bile (human and shark) according to Hammarsten, and thus interfere with the sulfur determination. But von Bergman could not detect any in dog's bile. It is evident that there are many sources of error in all the older methods.

The method outlined in this paper is not open to the criticisms of the methods previously used for the quantitative estimation of the bile salts. It is a simple procedure requiring careful technique only in washing out the various residues and making up to volume.

The results, although 6 to 8 per cent below the theoretical values, are very constant, as we have demonstrated over and over again when duplicates were run through by several different people. The determinations can be carried out so that the results are available within 8 hours.

SUMMARY.

A method is given for the quantitative estimation of the bile acid present in dog's bile. It is based on the fact that taurine gives up its NH_2 nitrogen quantitatively in the Van Slyke amino nitrogen apparatus. The taurocholic acid is hydrolyzed by NaOH into taurine and cholic acid. The amino nitrogen of the taurine is then determined by the gasometric method.

We are indebted to both Dr. Alice Rohde and Dr. Donald D. Van Slyke for their advice and help in working out this method; also to Dr. C. L. A. Schmidt for sulfur determinations.

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THE METABOLISM OF BILE ACIDS.

II. NORMAL FLUCTUATIONS IN HEALTHY BILE FISTULA DOGS.

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As a preliminary to any series of bile acid experiments it is necessary to establish the normal curve of excretion. This curve of excretion may vary with the condition of the animal, with the diet administered, and with other factors which may not be subject to control. Unless otherwise noted the dogs used in these experiments were in fine physical condition as shown by normal activity, vigorous appetite, a uniform weight curve, and normal blood picture. The operative procedure and general routine care of these animals have been described in detail in an earlier publication, Hooper and Whipple (1). It is very essential that these dogs be exercised, fed, set up, and drained regularly. Diet regulation is very important and at times difficult. Diarrhea is a troublesome feature which must be controlled, and this is often best done by a proper admixture of kaolin to the food. We have found it necessary to set up the dogs for collection of bile at least 30 minutes before the actual collection is started. This assures a complete drainage of the thick viscid night bile which escapes only slowly from the fistula. The presence of this concentrated bile in the first collection will cause high readings and introduce an error unless this precaution is taken. This explains some of the high readings in the first 2 hour periods of Tables VIII and IX. Unless otherwise stated, dogs were fed exactly 2 hours after the daily collection was started and again after the period of exercise following the collection. Weights were taken in the morning before the collection.

EXPERIMENTAL.

The method of chemical analysis has been controlled and shown to be reasonably accurate. The method of bile collection has been described and may be assumed to be accurately controlled. The dogs were kept in a room in which they were under constant supervision, which assured an accurate and complete bile collection. Occasionally a dog may be restless and displace the rubber tube in the fistula allowing the escape of bile, but the binders are large and by experience carefully fitted, so that such accidents are rare. When there was loss of bile the material was discarded unless the loss was very small or could be measured. Notes are made of any deviations from the uniform routine complete collection. As explained in another place we feel that 6 or 8 hour collections are more satisfactory than the longer 12 to 24 hour collections used by Stadelmann and other workers. The longer collections are more trying to the animal and usually cause loss of weight, appetite, and strength. This immediately introduces the factor of disease with its many unknown variables. We feel that these 6 hour collections continued over weeks and months in dogs which are in every respect healthy and active will give more truthful information about the normal bile acid metabolism.

It is to be noted in the first two tables (Tables VIII and IX) that the dogs were not drained for 30 minutes before the collections were begun. The first 2 hour periods show a bile which is more concentrated and contains more bile acids than does the second unit period. A part of this high excretion during the first 2 hours is undoubtedly to be explained by the presence of some of the concentrated night bile in the bile passages, not completely drained off before collections were begun. One must keep in mind the normal fluctuations in bile acid excretion on a mixed diet so that proper care may be exercised in the interpretation of the fluctuations noted under experimental conditions. Fluctuations in bile volume are at times startling and inexplicable. The constitution of the mixed diet may account for some of the fluctuations in bile volume. It has been pointed out elsewhere by Whipple and Hooper (4) that a meat diet produces a thin, pale, voluminous bile excretion, poor in bile pigments; and further

TABLE VIII.

Bile Acid Excretion—2 Hour Periods—Mixed Diet.

Dog 18–23. Simple Bile Fistula.

Date.	Hour.	Bile.	Amino nitrogen.		Taurocholic acid output.	Weight.	Remarks.
			Per cc. of bile.	Output.			
1918		*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Mar. 5	1–2	20	0.374	7.48			Mixed diet.
	3–4	26	0.231	6.00			
	5–6	29	0.158	4.58			
	1–6	75		18.06	664	32.0	
Mar. 6	1–2	17	0.344	5.85			
	3–4	26	0.228	5.93			
	5–6	21	0.198	4.16			
	1–6	64		15.94	585	32.0	
Mar. 7	1–2	25	0.253	6.32			Hb. 110 per cent.
	3–4	28	0.170	4.76			R. B. C. 6,290,000.
	5–6	32	0.114	3.65			
	1–6	85		14.73	541	33.3	
Mar. 8	1–2	22	0.264	5.80			
	3–4	34	0.190	6.46			
	5–6	25	0.191	4.77			
	1–6	81		17.03	626	34.0	
Mar. 11	1–2	34	0.247	8.40			
	3–4	32	0.160	5.11			
	5–6	25	0.189	4.72			
	1–6	91		18.23	670	32.8	
Mar. 12	1–2	23	0.253	5.82			
	3–4	27	0.148	3.99			
	5–6	19	0.153	2.90			
	1–6	69		12.71	467	34.0	
Mar. 13	1–2	28	0.368	10.30			
	3–4	29	0.368	10.67			
	5–6	25	0.225	5.63			
	1–6	82		26.60	978	35.0	
Mar. 14	1–2	21	0.315	6.61			
	3–4	21	0.286	6.60			
	5–6	18	0.300	5.40			
	1–6	60		18.61	662	35.5	
Mar. 15	1–2	34	0.390	13.26			
	3–4	22	0.277	6.09			
	5–6	26	0.223	5.80			
	1–6	82		25.15	923	34.8	

TABLE IX.

Bile Acid Excretion—2 Hour Periods—Mixed Diet.

Dog 17–151. Simple Bile Fistula.

Date.	Hour.	Bile.	Amino nitrogen.		Taurocholic acid output.	Weight.	Remarks.
			Per cc. of bile.	Output.			
1918		*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Mar. 5	1–2	7	0.828	5.79			
	3–4	26	0.675	17.55			
	5–6	28	0.329	9.21			
	1–6	61		32.55	1,196	42.5	
Mar. 6	1–2	11	1.26	13.86			
	3–4	23	0.549	12.62			
	5–6	14	0.379	5.30			
	1–6	48		31.78	1,167	41.8	
Mar. 7	1–2	24	0.409	9.81			Hb. 118 per cent.
	3–4	25	0.267	6.67			R. B. C. 6,350,000.
	5–6	31	0.213	6.70			
	1–6	80		23.18	852	42.5	
Mar. 8	1–2	13	0.618	8.03			
	3–4	6	0.816	4.89			
	5–6	13	0.717	9.32			
	1–6	32		22.24	818	43.0	
Mar. 11	1–2	13	0.437	5.68			
	3–4	15	0.278	4.17			
	5–6	10	0.101	1.01			
	1–6	38		10.86	400	41.5	
Mar. 12	1–2	13	0.780	10.14			
	3–4	13	0.780	10.14			
	5–6	4	0.717	2.86			
	1–6	30		23.14	850	43.0	
Mar. 13	1–2	10	1.00	10.00			
	3–4	3	0.691	2.07			
	5–6	15	0.663	9.94			
	1–6	28		22.01	809	42.5	
Mar. 14	1–2	32	0.656	20.99			
	3–4 / 5–6	8	0.457	3.65			2nd and 3rd collections combined.
	1–6	40		24.64	906	43.5	
Mar. 15	1–2	13	0.717	9.32			
	3–4 / 5–6	5 / 4	0.654	5.88			2nd and 3rd collections combined.
	1–6	22		15.20	559	44.0	

that a carbohydrate diet is associated with a thick, scanty bile excretion, rich in bile pigments. The mixed diet used in these experiments consists of a variable mixture of kitchen scraps containing meat, bones, bread, potato, rice, soup, etc.

Table VIII shows the variations of bile salt excretion from day to day in 2 hour collections. No preliminary drainage.

Table IX shows the variation in bile acid excretion from day to day in 2 hour collections. No preliminary drainage.

Hourly Variations in Bile Acid Elimination.

Dogs were set up for 8 and 9 hours to establish the hourly curve of daily excretion of bile acids. Compare Tables X and XI with Tables VIII and IX of this paper and subsequent tables in Paper III. These experiments are characteristic of many others which need not be tabulated at this time, but some of these supplementary observations will be given in later papers to prove other points.

TABLE X.

Bile Acid Excretion—Hour Periods—Mixed Diet.

Dog 18–137. Simple Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid per hour.	Remarks.
		Per cc. of bile.	Per hour.		
	cc.	*mg.*	*mg.*	*mg.*	
1	9.0	0.317	2.85	104	October 1.
2 and 3	20.0	0.302	3.00	110*	
4	9.0	0.274	2.47	90	
5	12.0	0.230	2.76	101	End of 5th hour fed mixed diet.
6	10.5	0.230	2.41	88	
7 and 8	22.0	0.216	2.37	87*	Hb. 90 per cent. R. B. C. 3,830,000.
9	10.5	0.230	2.41	88	Weight 24.0 lbs.

* Average of 2 hours.

Table X is quite typical of a group of experiments and shows a nearly uniform hourly elimination of bile acids. There is a general tendency for the bile acid excretion curve to fall slightly in the afternoon in spite of a midday feeding and careful preliminary drainage of the concentrated night bile before beginning the experiment.

TABLE XI.

Bile Acid Excretion—Hour Periods—Meat Diet.
Dog 17–34. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Tauro-cholic acid per hour.	Remarks.
		Per cc. of bile.	Per hour.		
	cc.	*mg.*	*mg.*	*mg.*	December 11.
1	3.8	0.686	2.61	96	Fed 500 gm. of meat.
2	9.1	0.438	3.98	147	" 300 " " "
3	9.1	0.351	3.19	118	Weight 29.8 lbs.
4	6.4	0.614	3.93	143	
5	4.1	0.614	2.52	92	
6	5.7	0.614	3.50	129	
7	5.1	0.658	3.36	124	
8	3.9	0.731	2.85	106	
9	6.0	0.686	4.10	150	

TABLE XI-a.

Bile Acid Excretion—2 Hour Periods—Fasting and Meat Diet.
Dog 17–34. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 2 hours.	Bile pigments in 2 hours.	Remarks.
		Per cc. of bile.	In 2 hours.			
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	February 13.
1–2	22	0.440	9.68	355	15.3	Fasting. Mixed diet day before fasting.
3–4	20	0.332	6.64	242	13.2	
5–6	20	0.318	6.36	235	13.9	Weight 31.8 lbs.
7–8	18	0.303	5.45	201	13.1	
9–10	19	0.318	6.04	220	7.0	
February 19 to 26—Rice, potato, and milk diet.						
						February 27.
1–2	21	0.189	3.96	146	11.0	Meat 250 gm. at beginning.
3–4	16	0.132	2.11	77	8.9	
5–6	20.5	0.161	3.30	121	12.4	Weight 27.5 lbs.
7–8	14	0.247	3.46	129	11.2	
9–10	13	0.261	3.39	124	11.8	

Tables XI and XI-a show slight fluctuations in bile acid output after feeding, but we do not attach any significance to this reaction. It will be noted (Table XI-a) that the level of bile

TABLE XII.

*Bile Acid Excretion Not Influenced by "Bile Exclusion."**
Dog 18–23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
June 3	62	0.326	20.20	741	6.5	30.2	No bile exclusion.
" 4	60	0.409	24.54	902	13.6	31.2	Mixed diet.
" 5	61	0.422	25.74	932	20.9	31.2	
" 6	82	0.371	30.42	1,115	11.2		
" 7	75	0.191	14.33	526	24.8	32.2	
" 8	58	0.284	16.57	608	16.2	32.0	Hb. 127 per cent. R. B. C. 6,665,000.
" 10	75	0.224	16.80	618	22.2	30.2	
" 11	77	0.289	22.25	817	28.7	31.7	
Average.....			21.4	782			No bile exclusion.
June 12	80	0.199	15.92	584	23.7	32.0	Absolute bile exclusion.
" 13	78	Lost.			16.3	32.0	Mixed diet.
" 14	96	0.346	33.22	1,215	20.0	32.0	
" 15	72	0.248	17.86	656	30.2		
" 16							Dog set up and drained 2 hrs.
" 17	66	0.180	11.88	436	24.1	30.5	
" 18	83	0.238	19.75	725	23.3	32.0	
Average.....			19.72	723			Complete bile exclusion.

* "Bile exclusion" means total inability of the dog to lick any bile from the fistula at any time. This is effected by means of a thick gauze pad and large binder.

acid excretion is influenced by the diet of the previous day. This point will be taken up again.

This method makes it possible to follow the hourly fluctuations in the bile acid output and to establish for the first time the actual hourly curve of bile acid elimination from hour to hour.

TABLE XIII.

Bile Acid Excretion Not Influenced by "Bile Exclusion."
Dog 18–23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	No bile exclusion.
May 21	57	0.223	12.72	467	12.9	30.75	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 22	52	0.255	13.26	487	10.8	30.75	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 23	73	0.323	23.58	866	12.9	30.50	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 24	61	0.253	15.44	567	12.9	30.25	Diet 300 gm. of cracker meal, 65 gm. of meat.
" 25–26						30.25	Mixed diet.
" 27	66					30.25	300 gm. of cracker meal, 65 gm. of meat.
" 28	72	0.221	15.90	574	7.5	30.50	300 gm. of cracker meal, 65 gm. of meat.
" 29	82	0.334	27.40	1,005	11.2	30.25	300 gm. of cracker meal, 65 gm of meat.
" 30							300 gm. of cracker meal, 65 gm. of meat.
" 31	72	0.210	15.12	552	10.0	30.00	
Average.....			17.6	645			No bile exclusion.
							Absolute bile exclusion.
June 19	77	0.252	19.40	712	18.8	32.50	300 gm. of cracker meal, 65 gm. of meat.
" 20	78	0.210	16.38	602	23.7	32.00	300 gm. of cracker meal, 65 gm. of meat.
" 21	59	0.294	17.35	637	19.8	31.50	300 gm. of cracker meal, 65 gm. of meat.
" 22	28	0.476	13.32	489	23.4	31.00	300 gm. of cracker meal, 65 gm. of meat.
Average.....			16.6	610			Complete bile exclusion.

The question of "bile exclusion" is a very important one for this entire series of experiments. The question resolves itself into the following: Does a bile fistula dog lick enough bile from

its fistula during the night to influence in any way the daily excretion of bile or bile acids? According to Stadelmann (3) in his experiments this "bile exclusion" is necessary. The exclusion of any possible ingestion of the dog's own bile in his experiments would cause a decrease of about ⅓ the total bile acid excretion. Because of our respect for Stadelmann's work we felt that it was necessary to control this point beyond the peradventure of a doubt. We submit a sufficient number of experiments in Paper V to prove that under the conditions of our experiments "bile exclusion" does not influence the output of bile acids. The reasons for this are discussed more in detail in that paper. These experiments (Tables XII and XIII) make the same point and are sufficient to inform the reader that this important factor has been properly controlled in experiments that follow. Absolute "bile exclusion" does not affect the output of bile acids on either a mixed diet or a known diet. The bile salt excretion can be partly controlled by diet, as will be shown in another paper. A diet rich in meat protein increases the output and a diet poor in meat protein reduces the excretion.

Effect of Bile by Mouth on the Following Day's Excretion.

In order to find out if bile by mouth in moderate amounts affected the following day's excretion several dogs were given bile at night and the bile acid excretion followed.

Table XIV shows that bile feeding in the late afternoon does not influence the following day's excretion of bile acids. Many other experiments have been performed with identical results. It is apparent from experiments tabulated in Paper III that the greater part (about 80 per cent) of the bile acids ingested as bile will appear in the bile fistula bile within 4 hours. We may assume that only bile ingested during the early morning hours (5 to 8 a. m.) influences the bile collections in our experiments. There is no clinical evidence that the dogs lick any bile from their fistulas during this period and the experimental data confirm this point.

TABLE XIV.

Bile Feeding at Night—Mixed Diet.
Simple Bile Fistula with Splenectomy.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
17–181	Average previous 10 days.			10.60	389		
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
	July 29						4 p.m. given 58 cc. of bile by stomach tube.
	" 30	36	0.267	9.61	352	25.50	5 p.m. given 60 cc. of bile by stomach tube.
	" 31	25	0.418	10.45	384	26.25	5 p.m. given 60 cc. of bile by stomach tube.
	Aug. 1	38	0.285	10.82	397	25.00	No bile given.
	" 2	37	0.244	9.03	332	25.25	Hb. 100 per cent. R. B. C. 4,500,000.
18–137	Average previous 10 days.			14.9	547		
	July 29					22.75	4 p.m. given 58 cc. of bile by stomach tube.
	" 30	50	0.310	15.50	569	23.50	4 p.m. given 60 cc. of bile by stomach tube.
	" 31	42	0.401	16.80	617	24.0	5 p.m. given 60 cc. of bile by stomach tube.
	Aug. 1	43	0.395	16.55	608	23.5	No bile given.
	" 2	30	0.145	4.35	159	22.75	Hb. 100 per cent. R. B. C. 4,425,000.

DISCUSSION.

Loeb (2) stated that the sulfur and nitrogen excretion in the bile is higher during the first 4 hours than during the second 4 hours after eating. From the tables given in this paper it is clear that the bile acid excretion is greater during the early part of the day than later in the afternoon. There is always a tendency to fall off during the 5th and 6th hours of the collection in spite of the ingestion of food. But in an individual the amount

excreted throughout the day is fairly uniform provided a fore-period of 30 minutes or longer for drainage of the bile fistula has been a part of the routine preceding the usual 6 hour collection.

It is evident that the amount of bile which these dogs normally lick from their fistulas during the hours they spend in their cages is insufficient to cause any variation in the following day's output (Tables XII and XIII). The only time our dogs are prone to lick their fistulas is just after the completion of the daily collection. But Table XIV shows that moderate amounts of bile at this time are excreted before the following day's collection is made, and do not affect the determination in any way.

SUMMARY.

Bile acid excretion in a healthy bile fistula dog given a mixed diet will show great variations from day to day.

The amount of bile acid excreted is usually somewhat higher in the morning than in the afternoon. This holds good even after complete drainage (½ hour) of the concentrated night bile, and in spite of liberal feeding 2 hours after collections are begun.

The amount of bile acid excreted hour by hour during any given day is fairly uniform.

The amount of bile which a dog may lick from its fistula during the afternoon and night resting period is not sufficient in our experiments to cause any demonstrable variation in the following day's excretion.

Moderate amounts of bile given by stomach in the late afternoon do not influence the following day's excretion of bile acids.

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THE METABOLISM OF BILE ACIDS.

III. ADMINISTRATION BY STOMACH OF BILE, BILE ACIDS, TAURINE, AND CHOLIC ACID TO SHOW THE INFLUENCE UPON BILE ACID ELIMINATION.

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The cholagogue action of whole bile given by stomach is a well known fact. It has been established by many experiments that the curve of bile volume excretion does not necessarily parallel the curve of excretion of bile pigments (Whipple and Hooper, 9). In other words it is possible to stimulate a free flow of bile which is poor in bile pigments, so much so that the total bile pigment output may fall to half normal during a period of active cholagogue excretion with twice normal output of bile. Hooper (3) has pointed out several factors which may contribute to this reaction. It is well to recall that taurocholic acid given by mouth has a marked cholagogue action—experiments given below add more data to establish this statement. An ether extract of dried bile, which of course contains a mixture of substances, has no cholagogue action, but inhibits the excretion of bile pigments. It is of considerable interest to know that certain substances can stimulate or inhibit the total bile excretion while influencing the output of the various bile constituents in the same or opposite direction.

In 1875 Socoloff (7) injected glycocholic acid into a dog and found an increased excretion but no increase in the per cent of bile acids. But his method was questionable and he used only one dog. Rutherford and Vignal (5) injected bile acids into the jejunum and found an increased output of bile acids, but they do not mention how the bile acids were estimated. To Schiff (6) is given the credit for having established the fact that there is a reabsorption of bile acid and this is often termed the "circulation

of the bile." This term "circulation of the bile" is used loosely by some writers and is at times misquoted to indicate a circulation of other of the substances found in the bile, for example, bile pigments. We have definite proof that bile pigments are not absorbed from the intestine (Hooper and Whipple, 4). At present we have little if any accurate knowledge about the many other substances present in fresh bile—they may be absorbed or not.

In giving dog's bile by mouth, Stadelmann (8) found that with doses of 2.0 or 2.5 gm. of bile salt, the cholagogue action lasted up to 24 or 36 hours, but the bile salt was excreted within 24 hours, usually within 10 hours. With 1.5 gm. of pure sodium glycocholate the whole amount was excreted within 12 hours. The greatest cholagogue action was during the first 6 hours. From then on it began to abate and was over within the next 12 hours. The bile salts may have been excreted in less than 10 to 12 hours, for Stadelmann performed bile salt analyses only upon these large collections.

With doses of 3 to 5 gm. morning and evening the salts and volume were tremendously increased but the excretion was not directly proportional to the amounts given. 5 gm. twice a day for 3 successive days kept both the volume and salts above normal for more than 8 days, even though they were continually decreasing. These experiments of Stadelmann's are in harmony with those tabulated below, and supplement our experimental data.

One is not surprised to observe in the experiments given below that the curves of whole bile excretion and bile acid excretion may run parallel after the oral administration of fresh dog's bile—moreover, on the contrary, that these curves may be widely dissociated. A moderate dose of whole dog's bile given by stomach will cause a distinct cholagogue action and a parallel increase in bile acids. With the fall in bile volume after 3 to 5 hours there is a fall in the bile acid output. It is possible to give sugar with concentrated bile by stomach tube and completely inhibit the cholagogue action while a great rise in output of bile acids is taking place. We believe this procedure gives a maximum concentration of bile acids in dog's bile, as high even as 7 to 9 per cent by weight. Perhaps this represents the maximum power of the liver cells to concentrate bile acids in whole bile, at least under the conditions of the experiment.

EXPERIMENTAL.

The first four tables (Tables XV to XVIII) are to be taken as a unit because they illustrate the uniformity of reaction which follows the ingestion of moderate amounts of fresh dog's bile. The taurocholic acid content varies from 0.63 to 1.83 gm. in any given single dose. The hourly curve of bile acid excretion is remarkably

TABLE XV.

Bile Feeding.

Dog 17–181. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					August 27.
1	8.0	0.319	2.55	93	End of 1st hour given 50 cc. of whole dog's bile containing 752 mg. of taurocholic acid.
2	18.5	0.667	12.30	452	
3	11.0	0.662	7.28	267	
4	7.0	0.596	4.17	153	
5	6.5	0.333	2.16	79	End of 5th hour fed mixed diet.
6	7.0	0.222	1.55	56	
7	10.5	0.222	2.33	85	
8	10.0	0.210	2.10	77	
9	9.0	0.207	1.86	68	Hb. 100 per cent. R. B. C. 4,810,000.
10	10.0	0.221	2.21	81	Weight 25.3 lbs.

constant and usually shows that the largest elimination takes place during the first 3 hours after ingestion. Some experiments show the maximum elimination of bile acids during the first hour, again during the second or third hour. The bile acid concentration per cc. of the bile eliminated usually closely parallels the curve of total excretion. The cholagogue action is marked with the larger doses but is practically absent following the smallest dose.

Table XV shows that about 90 per cent of bile acid in whole bile given by mouth is excreted within 3 hours.

TABLE XVI.

Bile Feeding.

Dog 18–137. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					September 5.
1	10.0	0.442	4.42	162	
2	7.5	0.313	2.35	86	End of 2nd hour 75 cc. of bile containing 628 mg. of taurocholic acid.
3	11.0	0.470	5.17	189	
4	9.0	0.666	6.00	220	
5	8.5	0.696	5.92	217	End of 5th hour fed mixed diet.
6	7.0	0.464	3.25	119	
7	10.0	0.275	2.75	101	
8	8.0	0.261	2.25	82	Hb. 120 per cent. R. B. C. 5,060,000.
9	8.0	0.248	1.98	72	Weight 22.25 lbs.
10	7.0	0.221	1.55	56	

TABLE XVII.

Bile Feeding.

Dog 18–23. Simple Bile Fistula.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					August 6.
1	7.5	0.364	2.73	100	End 1st hour given 100 cc. of bile containing 1.825 gm. of taurocholic acid.
2	22.0	0.757	16.65	612	
3	17.0	0.980	16.65	612	
4	20.5	0.925	18.95	696	
5	13.5	0.582	7.86	288	End of 5th hour fed mixed diet.
6	11.0	0.373	4.10	150	
7	10.5	0.276	2.90	106	Hb. 120 per cent. R. B. C. 6,375,000.
8	9.0	0.345	3.10	114	Weight 31.5 lbs.

TABLE XVIII.

Bile Feeding.

Dog 18–137. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					September 24.
1	8.5	0.530	4.51	165	
2	11.0	0.430	4.73	173	End of 2nd hour 100 cc. of bile containing 951 mg. of taurocholic acid.
3	18.0	0.788	14.18	520	
4	16.0	0.845	13.51	499	
5	10.0	0.516	5.16	189	End of 5th hour fed mixed diet.
6	10.5	0.508	5.33	195	
7	11.0	0.326	3.58	131	
8	11.0	0.312	3.43	126	Hb. 90 per cent. R. B. C. 3,830,000.
9	10.0	0.260	2.60	95	Weight 23.3 lbs.

TABLE XIX.

Concentrated Bile Feeding.

Dog 17–84. Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					October 15.
1	7.5	0.546	4.09	150	
2	6.4	0.445	2.85	104	End 2nd hour 170 cc. of concentrated bile solution* containing 11.50 gm. of taurocholic acid given by stomach tube.
3	21.0	0.968	20.31	745	
4	26.0	1.430	37.20	1,365	Vomited about 200 cc. of fluid.
5	29.0	1.360	39.44	1,445	End 5th hour fed mixed diet.
6	34.3	1.220	41.88	1,535	
7	54.0	1.000	54.00	1,980	
8	18.0	0.657	11.82	433	Hb. 108 per cent. R. B. C. 5,700,000.
9	12.0	0.299	3.59	132	Weight 31.5 lbs.
Oct. 16	60.	0.313	18.80	690	Usual 6 hour collection.
Oct. 17	64.	0.328	21.00	752	Usual 6 hour collection.
Oct. 18	56.	0.300	16.80	616	Usual 6 hour collection.

* Solution prepared by evaporating bile to dryness, extracting with water, and centrifuging. The clear supernatant fluid is this bile solution.

Table XVI shows that about 60 per cent of bile acid administered by mouth was excreted within 4 hours. Note little if any cholagogue action.

Table XVII shows that about 90 per cent of bile acid administered by mouth was excreted in 4 hours.

Table XVIII shows that about 85 per cent of bile acid given in bile by mouth is excreted within 4 hours.

TABLE XX.

Concentrated Bile Feeding.
Dog 18–137. Simple Bile Fistula and Splenectomy.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					September 18.
1	7.5	0.187	1.40	51	
2	12.0	0.330	3.96	145	End 2nd hour 370 cc. of bile solution† containing 11.60 gm. of taurocholic acid given by stomach tube.
3	16.5	0.729	12.00	440	Vomited 60 cc.
4	20.0	1.111	22.22	816	
5	16.0	1.200	19.20	704	End 5th hour fed mixed diet.
6	14.0	1.010	14.15	519	Hb. 120 per cent. R. B. C. 5,060,000.
7,8, and 9	45.0	0.750	11.24*	413*	Weight 23.5 lbs.

* Average of 3 hours.

† Solution prepared by evaporating bile to dryness, extracting with water, and centrifuging. The clear supernatant fluid is this bile solution.

Tables XIX and XX show remarkably well the intense reaction which may follow large doses of taurocholic acid by mouth. Whole bile was not given, but a crude watery extract of dried dog's bile which is rich in taurocholic acid. Vomiting occurred and the amount regurgitated is not known.

The cholagogue action is noted immediately and is sustained many hours and even days (Table XIX). The concentration of bile acids per cc. of bile is much above normal and the actual output of almost 2 gm. per hour is reached in one experiment. Large

amounts of bile acids seem to cause no ill effects, immediate or delayed, except some nausea and vomiting. What effect if any is produced in the general body metabolism is unknown.

Table XIX shows that bile acid fed by mouth in high concentration causes a great increase in bile acid output for 6 hours, and is a

TABLE XXI.

Concentrated Bile Feeding Plus Sugar.
Dog 15–22. Simple Bile Fistula.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 1 hour.	Remarks.
		Per cc. of bile.	In 1 hour.		
	cc.	*mg.*	*mg.*	*mg.*	
					September 26.
1	21.5	0.675	14.50	532	
2	22.0	0.380	8.36	301	End of 2nd hour 125 cc. of bile solution* containing 8.770 gm. of taurocholic acid—100 gm. of sugar-water. Little vomiting.
3	15.0	1.54	23.10	848	
4	9.5	1.93	18.35	673	
5	14.5	2.31	33.50	1,230	End of 5th hour fed mixed diet.
6	15.5	2.35	36.45	1,340	
7	15.0	2.26	33.90	1,245	
8	11.0	1.80	19.80	727	Hb. 110 per cent. R. B. C. 5,280,000.
9	8.5	1.77	15.05	552	Weight 32.8 lbs.
Sept. 27.	37.0	1.19	44.00	1,615	Usual 6 hour collection.
Sept. 30.	12.0	1.18	14.10	518	Usual 6 hour collection.

* Bile evaporated to dryness, the residue extracted with water, and centrifuged. The clear supernatant fluid is this bile solution.

cholagogue for a much longer period. About 65 per cent was excreted in 6 hours.

Table XX shows that concentrated bile acid by mouth may cause a long delayed bile acid excretion.

Table XXI shows that bile acid plus sugar by mouth causes a delayed bile acid excretion with fairly low volume of bile. The extreme cholagogue action of bile acids may be completely

inhibited by simultaneous administration of a sugar solution by stomach. Sugar solutions alone will cause the excretion of a very concentrated bile but will not modify the bile acid curve. This

TABLE XXII.

The Effect of Taurine and Cholic Acid Separately and Combined. Dog 17–151. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen. In 1 cc. of bile.	Amino nitrogen. In 6 hours.	Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Urinary N.	Remarks.
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	*gm.*	
Average 11 days.	15		8.0*	294			3.3	Hb. 138 per cent. R. B. C. 7,670,000.
Mar. 30.....	34	0.277	9.42	346	26.6	36.75	2.52	Before collection: 4 gm. of cholic acid in capsule.
" 31.....	29	0.133	3.86	142	19.4	36.60	3.25	
Apr. 1.....	18	0.312	5.61	206	23.5	36.50	2.41	Before collection: 0.75 gm. of taurine in 30 cc. of salt solution intravenously.
" 2.....	28	0.353	9.89	366	17.5	36.25	2.63	
" 3.....	45	0.797	35.85	1,316	17.3	35.80	2.63	Before collection: 3 gm. of mixture $\frac{3}{4}$ cholic acid, $\frac{1}{4}$ taurine in capsule.
" 4.....	19	0.467	8.88	326	18.2	35.75	2.30	
" 5.....	47	1.141	53.60	1,967	20.9	35.50	2.58	Before collection: 4 gm. of taurocholic acid in capsule.
" 6.....	15	0.402	6.03	220		35.00	2.52	Hb. 125 per cent. R.B.C. 6,980,000.

* This dog was kept on a diet of 75 gm. of cane sugar and 100 gm. of glucose during this entire experiment, as well as during the fore-period of 11 days.

experiment gives the highest concentration of bile acids per cc. of fistula bile (86 mg. per cc.). The sugar seems responsible for a definite delay in output of bile acids; but the output is enormous in spite of the low volume and slight delay in elimination.

Tables XXII and XXIII are companion experiments and show great uniformity of reaction. From these two and many other experiments it is established that taurine by mouth or intravenously does not in any way influence the output of bile acids. This is true during fasting periods as well as for full diets.

Taurocholic acid by mouth is known to be a cholagogue and this

TABLE XXIII.

The Effect of Taurine and Cholic Acid Separately and Combined. Dog 18–23. Simple Bile Fistula.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 6 hours.	Weight.	Urinary N.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	*gm.*	
Average 12 days.	24		8.7*	319			3.10	Hb. 120 per cent. R. B. C. 7,895,000.
Mar. 30.....	45	0.551	24.75	909	18.0	27.7	3.27	Before collection: 3 gm. of cholic acid in capsule.
" 31.....	34	0.183	6.22	228	24.6	27.4	3.25	
Apr. 1.....	40	0.142	5.68	208	18.8	27.1	2.57	Before collection: 0.75 gm. of taurine in 25 cc. of salt solution intravenously.
" 2.....	30	0.240	7.20	264	14.6	27.0	2.74	
" 3.....	55	0.698	38.4	1,300	10.5	26.6	2.55	Before collection: 2.6 gm. of mixture $\frac{3}{4}$ cholic acid, $\frac{1}{4}$ taurine in capsule.
" 4.....	29	0.354	10.25	376	11.1	26.5	2.69	
" 5.....	56	0.867	48.53	1,780	22.4	26.4	2.58	Before collection: 3 gm. of taurocholic acid in capsule.
" 6.....	37	0.171	6.33	232	14.8	25.8	3.30	Hb. 120 per cent. R. B. C. 7,300,000.

* This dog was kept on a diet of 75 gm. of cane sugar and 75 gm. of glucose during this entire experiment, as well as during the fore-period of 12 days.

action is well shown in our experiments. About 40 to 50 per cent of the taurocholic acid is excreted in the bile during the 6 hour collection period. There may have been slight delay in excretion owing to the use of capsules rather than solutions.

Taurine plus cholic acid fed by mouth exerts the same influence on the bile fistula dog as does the pure taurocholic acid. In other words this synthesis can take place in the body with great ease and rapidity. The cholagogue action and increased bile acid output are identical, whether the mixture of taurine plus cholic acid is given or the pure taurocholic acid.

Cholic acid alone has less than usual influence upon the bile acid output if given after a long fasting period. There may be a little increase in bile acid excretion or there may be a decided increase. The reaction seems to depend upon the amount of taurine present in the body which is available to combine with the cholic acid radical. Note additional data in Table XXIV.

Table XXIV furnishes more interesting data concerning the feeding of cholic acid and its influence upon the bile acid excretion. A small dose (2.0 gm.) of cholic acid acts as a cholagogue but does not increase the output of bile acid after an 11 day fasting period. A larger dose (4.0 gm.) of cholic acid after 9 days fasting produces a cholagogue action and a definite increase in the taurocholic acid of the bile—an increase of about 100 per cent. The next day the same dose repeated gives the same cholagogue action, but little if any increase in the bile acid output. We may assume that the first dose of cholic acid combined with all the available taurine in the body and formed taurocholic acid to be eliminated in the bile. The second large dose given the next day found no taurine to complement the cholic acid and no taurocholic acid resulted. We see therefore that either taurine or cholic acid can act as limiting factors in the over-production of taurocholic acid. This is particularly true of cholic acid, which is probably the normal determining factor. Cholic acid given by mouth during periods of full diet is usually associated with a strong cholagogue action and a great increase in output of bile acids (Table XXIV—Dog 17–34). This indicates an abundant exogenous source of taurine in the common mixed diet.

Table XXV shows the curve which results from the feeding of sodium taurocholate. Its reaction seems to be identical with that

TABLE XXIV.

Cholic Acid Feeding.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
15–22	Average.	16		10.0	367		Average 3 days after 11 days fasting.
	May 4	35	0.231	8.09	297	28.0	2.0 gm. of cholic acid in capsule at beginning.
	June 3	16	0.510	8.16	299	29.5	After 8 days fasting.
	" 4	38	0.535	20.33	746	29.0	4.0 gm. of cholic acid in emulsion at beginning.
	" 5	35	0.250	8.75	321	28.4	4.0 gm. of cholic acid in emulsion at beginning.
17–34	Oct. 9	24	0.537	12.90	473	31.5	Mixed diet.
	" 10	114	0.579	66.00	2,420	31.5	4.0 gm. of cholic acid in 15 cc. of alcohol at beginning.
	" 11	128	0.478	61.2	2,250	31.5	4.0 gm. of cholic acid in 15 cc. of alcohol at beginning.

TABLE XXV.

Bile Salt Feeding.

Dog 15–22. Simple Bile Fistula.

Hour.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Pigments in 2 hours.	Remarks.
		In 1 cc. of bile.	In 2 hours.			
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	
						September 4, 1917.
1–2	18	0.800	14.14	529	6.8	End 2nd hour 10 gm. of crude sodium taurocholate given by stomach.
3–4	58	1.711	99.26	3,640	18.4	
5–6	45	1.597	71.85	2,635	11.7	
7–8	22	0.642	14.12	518	8.4	Weight 35.0 lbs.

observed after taurocholic acid feeding. The cholagogue effect is pronounced and about 50 per cent of the taurocholate is recovered in the first 4 hours of collection. The crude taurocholate was not carefully prepared or analyzed, so that the actual percentage output cannot be estimated. The reaction subsides rapidly and falls almost to normal within 6 hours.

DISCUSSION.

Von Bergman (1) fed sodium cholate to dogs and found a decided increase in the sulfur content when only 1.0 gm. was given. But his dog weighed only 4.5 kilograms. A dose of 2.0 gm. caused an even greater increase, but did not double it. He also fed a dog of 8.5 kilograms sodium cholate (2.0 gm.) every day for 3 days. The first day there was a sufficient amount of sulfur excreted to account for all the cholate being changed into taurocholic acid. But each successive day the sulfur excreted was less. The third day it was not back to normal although it was much lower. He explains this by saying that the body could not furnish sufficient taurine to unite with the abnormally high amount of sodium cholate given. Our experiments show that a minimal amount of taurine is available after a long fasting period (Table XXIV). Our mixed diet was probably richer in sulfur than von Bergman's diet of 200 gm. of meat, 150 gm. of rice, and 30 gm. of casein. Note the high output with cholic acid feeding and a liberal mixed diet (Table XXIV).

Goodman (2) gave 0.6 gm. of cholic acid to a dog on one day and found a decided increase in the amount of bile and the cholic acid excreted. This experiment was done once on a dog of 4.5 kilograms weight on a diet of dog biscuit.

The *cholic acid* appears to be the important determining factor in the output of taurocholic acid, and it is of some importance to learn the source of cholic acid in the body, its true metabolic history, and its usefulness and ultimate fate in the body. The solution of these and other questions relating to cholic acid is not easy but will repay further investigation.

Feeding cholic acid causes a minimal reaction after long periods of fasting. Repeated doses of cholic acid during fasting result in complete failure of subsequent doses to call out an increase of bile acid excretion. This may be interpreted to mean that under

these conditions of fasting and repeated ingestion of cholic acid the available taurine is reduced close to zero. The cholic acid is unable to combine with taurine in the usual way and is eliminated in some other form, perhaps in the bile or elsewhere.

Feeding cholic acid without taurine in a liberal mixed diet will give a maximal cholagogue action and output of bile acids. Presumably under these conditions there is ample taurine to combine with the cholic acid. The result is a large increase in taurocholic acid in the bile. Probably under normal conditions of diet and health there is always available an excess of taurine so that the normal determining factor is the cholic acid radical. Depending upon the available supply of cholic acid there is a high or low output of bile acids in fistula bile. It is evident that certain foods favor a high bile acid output, and presumably furnish considerable amounts of cholic acid in their metabolic history. Much more data on this point will be furnished in other publications.

SUMMARY.

When moderate amounts of bile are given by mouth (less than 1.8 gm. of taurocholic acid) about 90 per cent of the contained taurocholic acid is excreted in the first 4 hours.

Larger amounts of concentrated bile (8.0 to 11.0 gm. of taurocholic acid) may prolong the cholagogue action for many hours or even days.

A large amount of concentrated bile given with sugar causes a very high concentration of bile acids in the bile excreted (7 to 9 per cent). There may even be an absence of cholagogue action. This may represent the maximum effort of the liver cell to concentrate bile acids in bile.

Taurocholic acid and sodium taurocholate given by mouth have the familiar cholagogue action and a large amount will appear in the bile fistula bile (40 to 80 per cent) within 4 to 6 hours, depending upon the dose given.

Taurine intravenously has no effect on the excretion of bile acids.

Taurine plus cholic acid by mouth causes a marked increase in bile secretion and bile acid output—as much as does taurocholic acid itself.

Cholic acid by mouth usually causes a distinct cholagogue effect. Cholic acid fed during long periods of fasting gives a minimal output of bile acids, but fed during full diet periods gives a maximal output of bile acids. This reaction probably depends upon the available supply of taurine, which is much reduced after fasting, but is abundantly available during full diet periods.

We are indebted to Dr. C. L. A. Schmidt for a large amount of taurine which was prepared in his laboratory.

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THE METABOLISM OF BILE ACIDS.

IV. ENDOGENOUS AND EXOGENOUS FACTORS.

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This paper gives the results of experiments to show the excretion of bile acids during periods of fasting, of sugar feeding, and of standard diets. The total daily urinary nitrogen excretion is given in some experiments to show that the output of bile acids runs an interesting parallel to the body's endogenous nitrogen metabolism. Limited diets may profoundly influence the curve of bile acid excretion, and the nitrogenous portion of the diet is most important. It is obvious that certain meat proteins added to the diet profoundly modify the excretion of bile acids. So there seems to be an interesting relationship between the metabolism of meat proteins from the food and of bile acids excreted in the bile. It is at least possible that a similar relationship may hold for the tissue proteins of the body and the bile acid excretion. Much more experimental data must be submitted but the experiments outlined below make certain fundamental points quite clear.

Bidder and Schmidt (1) followed the solid constituents of the bile and concluded that there was an increase on a meat diet. Spiro (3) followed the sulfur in the food and in the bile and found that increasing amounts of meat increased the sulfur excreted, but not proportionately. He also stated that there was a continuous sulfur excretion during fasting, but at a much lower level than when fed. Also, feeding carbohydrates decreases the amount of sulfur excreted. These experiments are in accord with our results.

Kunkel (2) followed the sulfur partition in a single day and found that the sulfur increased in the bile on starvation at one time, and not at another. Bread and milk decreased the sulfur while various amounts of meat did not have any effect at all. Twice that amount of blood by stomach decreased the bile sulfur. The dog lived only about a month after the experiment, so that very little importance can be attached to these experiments.

EXPERIMENTAL.

These dogs were kept on the usual routine already described for the previous articles of this series. The experiments given in Tables XXXI to XXXIV show the urinary nitrogen excretion

TABLE XXXI.

Sugar Feeding.

Dog 17–151. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*lbs.*	
Mar. 18	13	0.815	10.59	389	38.4		43.0	75 gm. of cane sugar, 50 gm. of glucose given daily by stomach tube.
" 19	16	0.618	9.88	363	69.5	4.76	40.7	Hb. 125 per cent. R. B. C. 6,840,000.
" 20	11	0.972	10.69	392	42.0	3.05	40.8	
" 21	10	0.802	8.02	294	20.9	3.16	39.7	No bile exclusion in this experiment.
" 22						3.89	38.7	
" 23	13	0.780	10.14	372	36.6	2.71	39.0	75 gm. of cane sugar, 100 gm. of glucose given daily by stomach tube.
" 24	12	0.482	5.78	212	37.6	3.33	38.4	
" 25	11	0.556	6.11	224	21.0	3.92	38.5	
" 26	15	0.379	5.68	209	26.1	3.42	38.0	
" 27	30	0.236	7.08	260	14.6	3.41	37.9	
" 28	20	0.279	5.58	205	29.2	3.17	37.25	Hb. 138 per cent. R. B. C. 7,670,000.
" 29	19	0.472	8.98	329	30.8	3.11	37.0	
Average.....			8.05	295		3.45		86 mg. of bile acid per 1.0 gm. of urinary nitrogen.

per 24 hour periods. In these experiments the dogs were kept at all times in standard metabolism cages arranged for complete collection of the urine with elimination of the feces. Diarrhea was never present and no fecal nitrogen is included in these figures. The night bile of course was included in urine collections, but the nitrogen concerned is constant and rarely exceeds 0.2 gm.

per 24 hours. The dogs were catheterized at the same hour each day, weighed, and given the sugar solutions or water by stomach tube. The cage was then washed out and the washings added to the cage urine, bladder urine, and bladder washings, which were made up to a unit volume. Duplicate specimens were analyzed by the Kjeldahl method and the total nitrogen calcu-

TABLE XXXII.

Fasting.

Dog 17–151. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*lbs.*	
April 23	38	0.513	19.50	716	13.2		40.7	No bile exclusion in this experiment.
" 24	18	0.510	9.18	337	41.3	2.24	38.6	Hb. 132 per cent. R. B. C. 6,800,000.
" 25	15	0.704	10.56	388	33.9	4.14	37.4	
" 26	15	0.508	7.62	279	24.9	3.97	36.6	
" 27	11	0.615	6.76	248	15.2	4.20	36.0	Set up 3 hours.
" 28	27				16.0	4.62	34.5	" " 3 "
" 29	25	0.543	13.68	502	13.1	4.20	35.0	
" 30	15	0.641	9.61	352	12.0	3.83	34.5	
May 1	18	0.711	12.78	468	12.5	3.55	34.13	
" 2	16	0.899	14.39	528	9.5	3.78	33.56	
" 3	15	0.676	10.15	373	11.8	3.97	33.13	Hb. 130 per cent. R. B. C. 7,235,000.
Average.....			11.42	419		3.85		109 mg. of bile acid per 1.0 gm. of urinary nitrogen.

lated. One of the dogs (15–22, Table XXXIV) has an obstruction in his urethra which makes catherization impossible. The regular 24 hour collections were made as usual, and the average of several days will correct for the daily variations which are in part due to variable amounts of bladder urine retained on different days.

Tables XXXI and XXXII are to be compared, as these experiments were performed upon the same healthy, vigorous dog under

identical experimental conditions. The amount of sugar given in the first experiment was not large for a dog of this size, and was increased from 125 gm. per day to 175 gm. during the last half of the experiment. It may be merely a coincidence, but during the second period of higher sugar intake the bile acid output fell somewhat. This dog averaged 3.45 gm. of urinary nitrogen per day and 295 mg. of taurocholic acid per 6 hours. The 6 hour amount of taurocholic acid per gm. of daily nitrogen is therefore 86 mg.

When this dog is put on fasting after a suitable resting period with liberal mixed diet we note a higher urinary nitrogen output, 3.85 gm. per day and 419 mg. of taurocholic acid per 6 hours. The amount per gm. of 24 hour nitrogen is therefore 109 mg. taurocholic acid per 6 hour period. As the endogenous urinary nitrogen excretion rises with fasting, as compared with sugar feeding, we note a parallel or slightly greater rise in the output of taurocholic acid.

We wish to point out an interesting fact in Tables XXXII and XXXIV. The first day in each table gives a remarkably high taurocholic acid figure, and this may not be clear until one remembers that a full mixed diet preceded this fasting period. The first day's bile acid output results from the mixed diet of the preceding day, but it is significant that after the first 24 hours the base line is reached and maintained. This is somewhat different from the basal nitrogen excretion, which does not reach its lowest level on fasting until the third or fourth day.

Tables XXXIII and XXXIV are like the preceding two experiments and correspond in almost every detail. These two experiments were done on two different dogs of approximately the same weight. Both were in excellent condition. The fasting dog of course shows a higher output of urinary nitrogen and also of taurocholic acid per 6 hour period. The fasting dog shows a urinary nitrogen of 3.76 gm. per 24 hours and taurocholic acid 407 mg. per 6 hours. The sugar fed dog presents a urinary nitrogen of 3.23 gm. per 24 hours and taurocholic acid 318 mg. per 6 hours. The amount of 6 hour taurocholic acid per gm. of 24 hour nitrogen is almost identical, 108 mg. in fasting and 98 mg. with sugar.

It seems sufficiently clear that there is a close relationship

between the endogenous nitrogen metabolism and the excretion of taurocholic acid in bile fistula bile. During fasting periods we may assume that more body protein is broken down and more taurocholic acid results from this process. We may assume that

TABLE XXXIII.

Sugar Feeding.

Dog 18–23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*lbs.*	
Mar. 18	52	0.204	10.60	389	26.2		33.3	75 gm. of cane sugar, 50 gm. of glucose given daily by stomach tube.
" 19	27	0.329	8.88	326	17.4	4.51	31.75	Hb. 118 per cent. R. B. C. 6,130,000.
" 20	31	0.324	10.04	368	15.6	3.72	31.06	No bile exclusion in this experiment.
" 21	17	0.507	8.62	316	10.1	3.08	30.75	
" 22	20	0.414	8.28	304	13.9	3.88	29.19	
" 23	22	0.288	6.33	232	10.7	3.22	29.75	75 gm. of cane sugar, 75 gm. of glucose given daily by stomach tube.
" 24	17	0.512	8.70	319	14.2	2.04	29.56	
" 25	23	0.394	9.06	333	22.0	3.64	29.44	
" 26	26	0.325	8.45	310	11.3	3.13	28.0	
" 27	33	0.194	6.40	235	12.6	3.19	28.56	
" 28	20	0.363	7.26	266	10.4	2.74	27.38	Hb. 120 per cent. R. B. C. 7,895,000.
" 29	34	0.332	11.30	415	14.2	2.38	27.06	
Average.....			8.66	318		3.23		98 mg. of bile acid per 1.0 gm. of urinary nitrogen.

sugar feeding enables the body to conserve its protein at the source, or enables the body to conserve its protein end-products and reconstruct these into body cells. When less bile acids are excreted during sugar periods we may wish to assume some such conservation of bile acid or its parent substance for other uses in

body metabolism. Refer also to Table XL, where there is even more conservation of bile acids or substances from which they are derived.

Fasting does not decrease the bile acid output to a remarkably low level. Sugar feeding causes a drop of bile acid excretion below the fasting level. These two points emphasize the fact

TABLE XXXIV.

Fasting.

Dog 15–22. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.					
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*lbs.*	
April 23	50	0.668	33.40*	1,225*	21.8		34.7	Dog not catheterized during experiment.
" 24	20	0.602	12.04	442	20.9	5.71	33.0	Hb. 150 per cent. R. B. C. 6,390,000.
" 25	27	0.470	12.68	465	32.9	3.75	32.38	No bile exclusion in this experiment.
" 26	18	0.486	8.77	322	21.9	3.16	31.5	
" 27	28	0.536	15.00	550	4.4	2.91	31.4	Set up 3 hours.
" 28	27	0.552	14.90	547	10.3	4.59	30.56	" " 3 "
" 29	22	0.493	10.85	398	4.2	3.58	30.20	
" 30	20	0.404	8.09	297	22.6	2.94	29.63	
May 1	17	0.505	8.58	315	26.6	3.19	29.20	
" 2	12	0.744	8.93	328	16.3	4.03	28.70	
Average.....			11.09	407		3.76		108 mg. of bile acid per 1.0 gm. of urinary nitrogen.

* Not included in average.

that there is an important endogenous factor in the bile acid metabolism.

Tables XXXV and XXXVI are identical in practically every respect and show a remarkable parallelism between the food intake nitrogen and the output of taurocholic acid. It will be shown later that this reaction depends in part upon the type of food protein. The first dog (Table XXXV) was in perfect condition during the entire experiment, maintained a constant weight,

TABLE XXXV.

Nitrogen in Food and Bile Acid Excretion.
Dog 17–151. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Jan. 7	43	0.403	17.32	636	21.4	38.0	Diet 375 gm. of cracker meal, 90 gm. of beef heart containing 100 calories and 0.5 gm. of nitrogen per kilo.
" 8	30	0.575	17.25	633	23.0	38.0	Hb. 135 per cent. R. B. C. 7,576,000.
" 9	49	0.246	12.05	442	24.7	38.0	
" 10	32	0.505	16.16	593	10.6	38.5	
" 11	28	0.311	8.70	319	6.3	38.5	
" 14	20	0.680	13.60	499	20.8	38.5	
" 15	65	0.260	16.90	620	20.1	39.0	No bile exclusion in this experiment.
" 16	28	0.766	21.44	787	18.5	39.0	
" 17	37	0.538	19.90	730	19.9	39.3	
" 18	17	0.785	13.35	490	16.0	38.8	Diarrhea.
" 21	16	0.604	9.66	354	18.9	39.0	
" 22	23	0.591	13.59	499	18.4	39.0	
Average.....			14.99	550	18.2		Diet 0.5 gm. of nitrogen per kilo.
Jan. 23	67	0.415	27.80	1,020	23.6	39.2	696 gm. of beef heart, 100 calories and 1.0 gm. of nitrogen per kilo.
" 24	50	0.460	27.00	994	14.8	39.5	
" 25	21	0.500	10.50	386	1.8	39.0	
" 28	37	0.675	24.97	918	3.9	39.3	
" 29	25	1.016	25.40	934	1.7	39.0	
" 30	32	0.898	28.73	1,055	2.8	39.3	
" 31	37	0.733	27.12	998	7.7	39.0	
Feb. 1	44	0.729	32.09	1,177	20.7	39.0	
" 4	51	0.645	32.89	1,206	8.0	39.0	
" 6	18	1.083	19.49	715	27.6	39.0	
" 7	40	0.871	34.84	1,279	7.7	38.8	
" 8	35	0.870	30.45	1,118	10.4	39.0	
Average.....			26.77	982	10.9		Diet 1.0 gm. of nitrogen per kilo.

TABLE XXXVI.

Nitrogen in Food and Bile Acid Excretion.
Dog 18–23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Jan. 7	42	0.272	11.02	405	40.9	33.0	Diet 325 gm. of cracker meal, 80 gm. of beef heart containing 0.5 gm. of nitrogen and 100 calories per kilo.
" 8	62	0.158	9.79	359	27.0	33.3	
" 9	72	0.073	5.22	191	25.0	33.0	Hb. 115 per cent. R. B. C. 7,024,000.
" 10	67	0.206	13.80	506	29.4	33.0	
" 11	69	0.158	10.93	401	17.6	32.8	No bile exclusion in this experiment.
" 14	67	0.143	9.58	453	20.8	33.0	
" 15	76	0.088	6.68	245	26.4	33.0	
" 16	56	0.211	11.81	433	14.6	33.5	
" 17	74	0.057	4.21	154	18.1	33.1	
" 18	70	0.184	12.85	472	12.2	33.3	
" 21	75	0.202	15.13	555	18.3	33.5	
" 22	68	0.226	15.40	566	19.9	33.5	
Average.....			10.53	395	22.5		Diet 0.5 gm. of nitrogen per kilo.
Jan. 23	79	0.3431	27.1	994	15.2	33.8	602 gm. of beef heart, 100 calories and 1.0 gm. of nitrogen per kilo.
" 24	77	0.246	18.93	685	18.8	33.6	
" 25	82	0.217	17.77	552	16.7	33.0	
" 28	43	0.392	16.85	618	11.0	32.0	350 gm. not eaten. Diarrhea.
" 29	58	0.416	24.12	885	9.1	31.8	250 " " " "
" 30	55	0.489	26.89	986	22.0	30.5	110 " " "
" 31	70	0.424	29.68	1,089	20.7	30.5	30 " " " "
Feb. 1	68	0.380	25.84	948	18.2	30.3	
" 4	80	0.312	24.96	916	18.1	29.5	Diarrhea.
" 5	60	0.395	23.70	870	14.6	29.0	" Noon meal omitted.
" 6	48	0.317	15 21	558	8.4	29.5	
" 7	74	0.213	15 76	578	14.1	29.0	
" 8	47	0.307	14.42	529	11.5	28.5	
Average.....			21.63	794	15.2		Diet 1.0 gm. of nitrogen per kilo.

and ate all food. The bile pigment figures are included and show remarkable and inexplicable fluctuation, particularly with the beef heart diet.

The taurocholic acid output is quite uniform each day with occasional fluctuations. The average daily output of bile acid per 6 hour period shows a very remarkable increase when the dog was suddenly changed from the diet rich in carbohydrate (0.5 gm. of nitrogen per kilo) to the beef heart diet (1.0 gm. of nitrogen per kilo). The increase in bile acid corresponds to the increase in food nitrogen—that is, about 100 per cent increase. The sharp rise in taurocholic acid following the change to a rich protein diet (beef heart) is well shown in both these experiments. The reaction even goes above the average figures of taurocholic acid output on the very first day of rich protein diet.

The second dog (Table XXXVI) shows a general reaction which is in every respect similar to the preceding experiment but for some diarrhea in the beef heart period. Associated with this was some loss of weight and appetite. These abnormal factors, however, did not influence the uniform reaction to the change in food protein. Additional data which confirm these experiments will be found in Paper V of this series.

Tables XXXVII and XXXVIII add some interesting data to that of the preceding experiments. In both these experiments the food nitrogen was decreased to 0.25 gm. per kilo by decreasing the cracker meal and replacing the beef heart with fat. The caloric value was held unchanged at 100 calories per kilo. One dog (Table XXXVII) showed practically no reaction to this change in diet and excreted almost the same amount of taurocholic acid as formerly upon a diet of 0.5 gm. of nitrogen per kilo. The other dog (Table XXXVIII) showed a decided drop in excretion of taurocholic acid but not to one-half the output on the diet containing 0.5 gm. of nitrogen per kilo. The level of bile acid output on this cracker meal-fat diet approaches the fasting excretion level of bile acid. This shows at once that there is no hard and fast parallel between the nitrogen intake and bile acid excretion, but it is clear that there is an important exogenous factor.

The mixed diet periods show a level of bile acid excretion which is below that of the beef heart diet. The elements in the

TABLE XXXVII.

Nitrogen in Food and Bile Acid Excretion.
Dog 17–151. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Feb. 11	32	0.525	16.80	617	38.8	40.0	262 gm. of cracker meal, 10 gm. of butter, 30 gm. of lard, 105 gm. of cane sugar, 0.25 gm. of nitrogen, and 100 calories per kilo.
" 12	30	0.536	16.08	590	28.2	39.5	
" 13	11	0.602	6.62	243	10.4	39.8	Hb. 125 per cent. R. B. C. 6,240,000.
" 14	32	0.425	13.60	499	20.6	40.0	
" 15	24	0.527	12.66	465	20.9	40.0	
" 18	32	0.385	12.32	452	10.0	40.0	No bile exclusion in this experiment.
" 19	20	0.493	9.86	362	13.2	40.0	
" 20	45	0.345	15.52	570	14.9		
" 21	23	0.695	15.98	586	5.2		
Average.....			13.27	487	18.0		Diet 0.25 gm. of nitrogen per kilo.
Mar. 5	61	0.534	32.55	1,196	16.6	42.5	Mixed diet.
" 6	48	0.662	31.79	1,167	21.5	41.8	
" 7	80	0.290	23.19	852	36.9	42.5	Hb. 118 per cent. R. B. C. 6,350,000.
" 8	32	0.695	22.25	818	10.1	43.0	
" 11	38	0.286	10.86	399	21.9	41.5	
" 12	30	0.771	23.14	850	3.9	43.0	
" 13	28	0.786	22.00	809	11.3	42.5	
" 14	40	0.616	24.64	906	5.8	43.5	
" 15	22	0.691	15.20	559	12.6	44.0	
Average.....			22.85	839	15.6		Mixed diet.

TABLE XXXVIII.

Nitrogen in Food and Bile Acid Excretion.
Dog 18–23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Feb. 11	48	0.231	11.08	407	18.4	30.3	30 gm. of lard, 59 gm. of cane sugar, 198 gm. of cracker meal, 10 gm. of butter, 0.25 gm. of nitrogen, and 100 calories per kilo.
" 12	43	0.174	7.47	274	16.1	29.5	
" 13	68	0.100	6.80	253	16.6	29.5	Hb. 115 per cent. R. B. C. 7,928,000.
" 14	58	0.027	1.60	58	18.2	29.3	
" 15	63	0.129	8.10	297	18.4	29.3	No bile exclusion in this experiment.
" 18	35	0.114	3.97	145	13.4	29.0	
" 19	64	0.197	12.64	464	11.1	29.2	
" 20	55	0.172	9.48	348	19.1		
" 21	51	0.085	4.34	159			
Average.....			7.27	267	16.4	Diet 0.25 gm. of nitrogen per kilo.	
Mar. 5	75	0.241	18.06	664	31.8	32.0	Mixed diet.
" 6	64	0.249	15.93	585	36.3		
" 7	85	0.173	14.73	541	37.0	33.3	Hb. 110 per cent. R. B. C. 6,290,000.
" 8	81	0.210	17.04	626	37.5	34.0	
" 11	91	0.200	18.22	670	27.9	32.8	
" 12	69	0.184	12.72	467	29.0	34.0	
" 13	82	0.324	26.60	978	25.3	35.0	
" 14	60	0.300	18.01	662	27.6	35.5	
" 15	82	0.307	25.14	923	29.5	34.8	
Average.....			18.50	679	31.3		Mixed diet.

mixed diet are of course variable. This diet is a mixture of kitchen scraps—bones, meat, bread, potato, rice, etc.

Table XXXIX shows a third dog which was placed upon these same diets containing 0.5, 1.0, and 0.25 gm. of nitrogen per kilo. This dog remained in perfect condition during the entire experiment and adds confirmatory data to the other experiments. This bile fistula dog has been under observation for 3 years, and it is known that there is a small communication between the common duct and duodenum. This allows a small amount of bile to enter the duodenum when the fistula is not draining freely, for example at night. This dog shows only a 50 per cent rise in bile acid output when the diet is changed from 0.5 to 1.0 gm. of nitrogen per kilo. Further change in diet from 1.0 to 0.25 gm. of nitrogen per kilo causes a fall from 1,262 mg. of taurocholic acid to 684 mg. per 6 hour period. These fluctuations are not proportional to the nitrogen content of the diets, but it is obvious that a rise in the food nitrogen intake does cause a rise in the taurocholic acid excretion and *vice versa*.

Table XL gives the data on two experiments which show the influence of a preceding period of fasting upon subsequent excretion of bile acids with a standard diet of 0.5 gm. of nitrogen per kilo. It is to be recalled that these same two dogs on a previous occasion showed an output of bile acids on this same diet which was much higher (550 and 875 mg. of taurocholic acid per 6 hour period—Tables XXXV and XXXIX). Compare with this high output the low excretion in Table XL on the same diet (376 and 419 mg. of taurocholic acid per 6 hour period). The only factor which can explain this difference is the preceding fasting period of 10 and 11 days. This low level of excretion is actually that of the fasting period or even lower. It is evident that the fasting period has caused a changed reaction in the body so that much less bile acid is permitted to escape in the bile. It may be a correct assumption that some of the material which under usual diet conditions goes to form the bile acid fraction is deviated for other uses in the body.

This reaction must be kept in mind whenever any diet experiments are planned for these bile fistula dogs. For certain experiments it might be assumed that a preliminary period of fasting might give an ideal simple base line from which to estimate the change brought about by a given diet. But the reaction may be

TABLE XXXIX.

Nitrogen in Food and Bile Acid Excretion.

Dog 15–22. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Jan. 7	13	0.835	10.85	398	10.0	32.0	325 gm. of cracker meal, 75 gm. of beef heart. 100 calories and 0.5 gm. of nitrogen per kilo.
" 8	20	0.992	19.84	728	3.5	32.3	
" 9	32	0.662	21.18	778	3.3	32.3	
" 10	26	0.591	15.36	551	3.6	32.5	No bile exclusion in this experiment.
" 11	38	0.373	14.17	520	7.3	32.3	
" 14	35	1.071	37.48	1,375	8.5	32.3	Large amount not eaten.
" 15	34	0.751	25.53	936	4.1	32.0	100 gm. not eaten.
" 16	14	0.920	12.88	473	3.4	32.0	100 " " "
" 17	46	0.630	28.98	1,065	6.4	32.5	150 " " "
" 18	43	0.822	35.35	1,297	7.5	32.0	
" 21	60	0.759	45.54	1,670	4.2	32.3	25 " " "
" 22	42	0.453	19.00	698	9.5	32.0	
Average.....			23.84	875	5.9		Diet 0.5 gm. of nitrogen per kilo.
Jan. 23	38	0.915	34.77	1,277	5.3	32.0	571 gm. of beef heart containing 100 calories and 1.0 gm. of nitrogen per kilo.
" 24	45	0.750	33.75	1,238	8.6	31.5	
" 25	62	0.700	43.40	1,593	5.4	31.0	
" 28	35	0.566	19.81	727	24.2	30.5	Diarrhea.
" 29	22	1.400	30.80	1,130	10.3	30.8	
" 30	30	1.730	51.90	1,905	7.8	31.0	
" 31	46	1.100	50.60	1,855	11.2	30.8	
Feb. 1	28	1.394	29.03	1,065	6.8	30.8	
" 4	29	1.131	32.80	1,205	13.6	31.0	
" 5	39	1.015	39.58	1,453	6.0	30.8	Noon meal omitted.
" 6	12	1.896	22.75	835	6.3	31.0	
" 7	42	0.698	29.31	1,075	6.6	30.5	
" 8	29	0.980	28.42	1,042	11.6	30.3	
Average.....			34.37	1,262	9.5		Diet 1.0 gm. of nitrogen per kilo.

TABLE XXXIX—*Concluded.*

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Feb. 11	29	1.105	32.05	1,175	11.6	32.0	209 gm. of cracker meal, 10 gm. of butter, 30 gm. of lard, 65 gm. of cane sugar, 0.25 gm. of nitrogen and 100 calories per kilo.
" 12	23	0.654	15.04	550	16.0	31.3	
" 13	47	0.403	18.95	696	8.4	32.0	
" 14	23	0.633	14.56	534	36.0	31.5	Hb. 124 per cent. R. B. C. 5,632,000.
" 15	48	0.658	31.58	1,160	10.8	31.8	
" 18	45	0.296	13.32	488	24.3	30.5	
" 19	28	0.331	9.26	340	14.6	30.5	
" 20	12	1.410	16.92	620	13.0	31.0	
" 21	17	0.922	14.97	599	17.2	31.3	
Average.....			18.50	684	16.8		Diet 0.25 gm. of nitrogen per kilo.

very different toward the same diet factor depending upon whether a fasting period or a carbohydrate diet period had preceded. To get a complete understanding of a single diet factor it will be necessary to observe any change in the bile acid excretion curve which may be associated with the administration of any such substance after short fasting periods as well as after carbohydrate or high protein diet periods.

Table XLI gives data to indicate that the formation of bile acids depends in part upon the functional capacity of the liver. The Eck fistula liver is produced by an anastomosis between the portal vein and vena cava and a ligature on the portal vein above this anastomosis which limits the blood supply of the liver to the hepatic artery and deviates all of the portal blood directly into the vena cava. The Eck fistula liver is known to be smaller than normal, to exhibit a moderate degree of fatty degeneration, to show a marked decrease in production of bile pigments (Whipple and Hooper, 4), and to present a distinct impairment of its normal capacity to

TABLE XL.

Nitrogen in Food and Bile Acid Excretion after Fasting Period.

Dog No.	Date.	Volume.	Amino nitrogen. In 1 cc. of bile.	Amino nitrogen. In 6 hours.	Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
17–151	Average 10 days fasting			11.8	433			
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
	May 4	37	0.454	16.80	616	11.0	32.6	Diet 330 gm. of cracker meal, 75 gm. of beef heart, containing 0.5 gm. of nitrogen and 100 calories per kilo.
	" 6	19	0.349	6.64	224	1.6	36.25	
	" 7	20				2.4	35.5	
	" 8	27	0.367	9.91	364	3.8	35.8	Hb. 130 per cent. R. B. C. 7,235,000.
	" 9	25	0.350	8.75	321	6.1	34.7	
	" 10	24	0.407	9.77	359	6.7	35.0	
Average				10.37	376			Diet 0.5 gm. of nitrogen per kilo.
15–22	Average 11 days fasting			11.09	407			
	May 6	21	0.587	12.32	452	2.9	29.2	Diet 285 gm. of cracker meal, 60 gm. of beef heart, containing 0.5 gm. of nitrogen and 100 calories per kilo.
	" 7	32				5.6	29.5	
	" 8	36	0.353	12.70	466	9.4	29.2	Hb. 125 per cent. R. B. C. 7,335,000.
	" 9	29	0.365	10.58	388	6.1	29.0	
	" 10	24	0.421	10.10	371	4.2	29.2	
Average				11.42	419			Diet 0.5 gm. of nitrogen per kilo.

excrete phenoltetrachlorphthalein (Whipple, Peightal, and Clark, 5). We may assume that the Eck fistula liver is functionally deficient. The observations in Table XLI harmonize with those

TABLE XLI.

Nitrogen in Food and Bile Acid Excretion. Eck Fistula. Dog 16–15. Simple Bile and Eck Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Jan. 7	16	0.314	5.03	183	4.5	20.5	Diet 200 gm. of cracker meal, 55 gm. of beef heart, containing 0.5 gm. of nitrogen and 100 calories per kilo.
" 8	25	0.258	6.45	236	5.7	20.0	Hb. 99 per cent. R. B. C. 6,240,000.
" 10	12	0.219	2.62	96	1.9	19.8	Not eating well.
" 11	16	0.144	2.30	84	5.3	19.8	" " "
" 14	12	0.155	1.86	68	3.3	20.0	
" 15	6	0.116	0.69	25		20.2	
" 16	10	0.141	1.41	52	1.6	20.7	
" 17	14	0.330	4.62	169	3.2	19.8	
" 18	21	0.273	5.73	210	3.7	20.3	50 gm. not eaten.
" 21	20	0.310	6.20	227	2.1	20.0	
" 22	20	0.212	4.24	155	2.1	20.0	
Average.....			3.74	137	3.34		Diet 0.5 gm. of nitrogen per kilo.
Jan. 24	30	0.447	13.41	492	3.7	19.8	Diet 355 gm. of beef heart, containing 1.0 gm. of nitrogen and 100 calories per kilo. 1 gm. of yeast twice a day.
" 25	17	0.534	9.07	333	0	20.8	

outlined above and show that an Eck fistula dog upon a standard diet will excrete not over one-half the normal amount of bile acids.

Compare this dog (Table XLI), weight 20 lbs. and output on standard diet of 137 mg. of taurocholic acid per 6 hour period, with the dog of Table XXXV, weight 40 lbs. and output on the same

standard diet of 550 mg. of taurocholic acid per 6 hour period. Also compare with dog of Table XXXIX, weight 33 lbs. and output on the same standard diet of 875 mg. of taurocholic acid per 6 hour period.

When the Eck fistula dog was changed to a rich protein diet we observe a considerable jump in the output of bile acids, as in the other dogs. It is not possible to keep an Eck fistula dog on a pure beef heart diet for any length of time without precipitating the characteristic Eck fistula intoxication which usually results in death.

DISCUSSION.

There is sufficient experimental data given above to make the point that both endogenous and exogenous factors are concerned in the metabolism of bile acids. There is a reasonably constant output of taurocholic acid during fasting periods, and this output may be somewhat diminished by administration of pure carbohydrate. The relative diminution of urinary nitrogen and taurocholic acid excretion may show a certain similarity under these experimental conditions. This may indicate a certain relationship between the metabolism of the body protein and the production of taurocholic acid. There is obviously a very important endogenous factor in the metabolism of bile acids.

It is equally clear that there is an important relationship between the output of taurocholic acid and the intake of certain food proteins. On certain diets a uniform level of bile acid excretion may be observed for days and a sudden shift to a similar diet containing double the amount of food nitrogen may cause a sudden doubling of bile acid excretion. This fact comes out clearly in several experiments in this paper but we wish to refer to additional evidence submitted in Paper V of this series. It is certain that some food proteins act very differently from others as to their value in modifying the bile acid excretion.

It is to be noted that the increased excretion of bile acids appears very promptly when the diet is changed to beef heart. It may be that the formation of bile acids by the liver cell is an automatic response to the proper stimulus, just as these cells respond to a protein digestion stimulus by urea formation. In the case of urea formation we know that even in the greatest food

shortage emergency the liver will form urea from amino-acids, even though the body may need all the food amino-acids, for example, after long periods of fasting. In the case of taurocholic acid we have shown that long periods of fasting followed by high protein feeding will show little rise in the taurocholic acid output contrary to what we might expect if this substance was purely a waste product to be eliminated from the split products of protein digestion. The body evidently conserves the taurocholic acid or its parent substance under certain conditions, perhaps for use elsewhere in the body in the reconstruction of its depleted body protein.

It is generally accepted that bile acids are dependent upon the normal liver cell function for their production. There has been little dispute in medical literature concerning this point. Yet there is very little available direct proof of this statement if one wished to argue that the bile acids were formed elsewhere in the body and eliminated in the bile. For example there is convincing evidence that bile pigments may be formed outside of the liver and excreted in the bile secondarily (Whipple and Hooper).

We have evidence in the Eck fistula experiment given above that bile acid output will be much subnormal in the Eck fistula liver which is functionally subnormal. This indicates that in a general way the bile acid output may fall with impaired functional capacity of the liver cell. Other experiments in which the liver is injured by chloroform and other poisons give similar results which will be published in the near future. All this evidence gives some direct and positive proof that the bile acids are actually produced by liver cell activity.

SUMMARY.

There is a uniform excretion of taurocholic acid in the bile fistula dog during fasting periods.

There is a uniform and slightly lower excretion of taurocholic acid in the same dog during similar periods of carbohydrate (sugar) feeding. This fall in taurocholic acid excretion is much like the fall in urinary nitrogen excretion under the same conditions.

There is an important endogenous factor in the bile acid metabolism and this may concern the body protein metabolism.

The output of bile acid in bile fistula bile may be influenced at will by suitable control of the diet. Meat protein seems to be of the greatest importance and a pure meat diet will give the highest output of bile acid per 6 hour period.

There is an important exogenous factor in bile acid metabolism which is concerned especially with the food protein.

After a long fasting period the bile fistula dog will not react to a high protein diet with the usual high bile acid output. There is evidently a deviation of certain precursors of the bile acid to serve other purposes in the body—perhaps to supply some important substances relating to body protein which have been depleted by the fasting period.

A functionally deficient liver (Eck fistula) produces less than one-half the normal amount of bile acid during a standard diet period. This is direct evidence (of which there has been little available) that the bile acids are formed essentially by liver cell activity.

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THE METABOLISM OF BILE ACIDS.

V. CONTROL OF BILE INGESTION AND FOOD FACTORS.

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The experiments tabulated below serve two purposes. They furnish additional data to make absolutely certain that the amount of bile acid excreted can be increased with the increase of meat protein nitrogen in the diet. These experiments in addition show that absolute exclusion of every drop of bile by mouth does not modify the excretion of bile acids under the conditions of the experiment. It was necessary to submit these control experiments because Stadelmann, who has done exceptional experimental work in this field, has stated that bile exclusion will cause a fall of bile acid excretion. He assumes therefore that a muzzle at night is necessary for accurate work, and that the bile fistula dogs lick sufficient bile from their fistulas to modify the following day's excretion of bile and bile acids.

Our dogs are set up each morning after a period of exercise followed by a 30 minute period of free drainage before collections are started. After the collection period of 6 hours the dogs are allowed to exercise in the yard before being fed in their cages. They are kept during the night in large cages of wire mesh beneath which are pans for the collection of excreta. The dogs, of course, during the night drain bile from their fistulas into the pans, but no bile collects where the dogs have access to it because the wire mesh retains no fluids. The dogs usually lick their fistulas when the collection is finished but it seems to be due in part to the fact that the skin itches where it is in contact with the abdominal binder. These dogs rarely lick bile from their fistulas in the early morning and at this time the flow is at a minimum. We feel that the experiments given make it quite clear that under

this laboratory routine the amount of bile which may be obtained by a given dog licking its own fistula is not sufficient to modify the excretion curve of bile acids.

We cannot attempt to explain Stadelmann's results, but two factors may be concerned. It is well known that dogs which have had bile fistulas for many months or years do not lick their fistulas as much as do dogs who are less accustomed to the bile fistula. One of our dogs has been under observation with a bile fistula for $3\frac{1}{2}$ years. Furthermore, in our cages, as stated above, bile could not accumulate during the night where the dog had access to it at any time, as the bile flowed through the mesh cage floor. If bile accumulated on the floor of a room or cage it is very probable that a dog would lick some of this bile in the morning and obtain a sufficient amount to make a decided difference in the output of bile acids.

It can be deduced from the experiments given in Paper III that moderate amounts of bile given late in the afternoon will not influence the output of bile acids on the following day. Bile given in the forenoon will cause a cholagogue action lasting several hours, but usually a rapid elimination of the excess of bile acids within 6 hours—at least 80 to 90 per cent elimination within this time.

EXPERIMENTAL.

Bile exclusion in these experiments indicates that the dog in question was unable to gain access at any time to any bile either from its own fistula or elsewhere. During the routine 6 hour collection all the bile is collected in a small rubber bag. At the end of the collection a muzzle is put securely on the dog, which is then permitted to run in the yard for a few minutes under careful observation. The dog is then brought in and dressed with its night binder which is worn until the next morning. The muzzle was not worn during the night except in one instance, where the dog tried to chew off the straps of the abdominal binder. The night binder was made of light canvas and fitted to the individual to cover the thorax and abdomen completely. Anteriorly it was held from slipping backward by soft webbing which encircles the neck and fore legs. The binder was held about the abdomen by soft webbing or straps. A large gauze pad was

placed over the bile fistula and served to absorb all the night bile. With this routine we are absolutely certain that no bile was ingested at any time during periods of "bile exclusion." The animals were comfortable and maintained their usual conditions of diet and activity.

Tables XLII, XLIII, and XLIV are to be considered as a unit. These three experiments were done at the same time under identical conditions, and the results are remarkably uniform. They are to be compared with Tables XXXV, XXXVI, and XXXIX in Paper IV of this series. In each of the three experiments tabulated (XLII to XLIV) the dog was placed upon a diet of cracker meal and beef heart containing 0.5 gm. of nitrogen and 100 calories per kilo. This diet with complete bile exclusion obtained for 1 week, and the average daily output per 6 hours shows a fairly uniform figure of 400 to 500 mg. of taurocholic acid per 6 hour period. The dog which is slightly heavier shows a slightly greater output.

The three dogs kept on this same diet for the second week were not prevented from licking their own fistulas in the cage during the night. It will be seen that the bile acid output remains about the same—it is actually somewhat less during this second week, 340 to 440 mg. of taurocholic acid per 6 hours.

During the third week each dog was given the first day with no bile exclusion, the second and third day with complete bile exclusion, and the fourth day with no bile exclusion. Individual fluctuations appear but this week in general agrees with the data of the first 2 weeks.

The fourth week was continued with strict bile exclusion but the diet was changed to a mixture of beef heart and a little cracker meal, giving 1.0 gm. of nitrogen and 100 calories per kilo. The figures from this group of experiments resemble those referred to in Paper IV. The increase in beef heart stimulates the output of bile acids from a level of 400–500 mg. to 700–900 mg. per 6 hours.

Two points are made by these and other experiments. Bile exclusion does not modify the excretion of bile acids under the conditions of our experiments. Certain food proteins in the diet have a marked influence on the excretion of bile acids in bile fistula bile.

TABLE XLII.

Known Diet With and Without Bile Exclusion.
Dog 18–93. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Aug. 5	57 ±	0.256	14.59 ±	536 ± *	31.8	Absolute bile exclusion. Diet 330 gm. of cracker meal. 60 gm. of beef heart = 0.5 gm. of nitrogen and 100 calories per kilo.
" 6	46	0.320	14.70	540	31.5	
" 7	38 ±	0.254	9.66 ±	355 ± *	32.0	
" 8	56	0.238	13.33	489	32.0	
" 9	59	0.255	15.05	552	32.3	
Average			13.50	494		Complete bile exclusion.
Aug. 12	54	0.224	12.08	443	30.8	No bile exclusion.
" 13	61	0.210	12.81	470	31.5	
" 14	58 ±	0.242	14.05 ±	516 ± *	31.5	
" 15	61	0.158	9.64	354	31.3	
" 16	64	0.181	11.58	425	31.3	
Average			12.00	442		No bile exclusion.
Aug. 19	70	0.266	18.62	683	31.0	No bile exclusion.
" 20	71	0.196	13.90	510	30.5	Bile exclusion 12 hours previously.
" 21	64	0.230	14.72	540	30.8	Bile exclusion.
" 23	77	0.185	14.25	523	30.5	No bile exclusion.
Aug. 28	72	0.326	23.48	862	30.0	Absolute bile exclusion. Diet 467 gm. of beef heart, 100 gm. of cracker meal = 1.0 gm. of nitrogen and 100 calories per kilo.
" 29	72	0.293	21.08	774	29.8	
" 30	76	0.335	25.48	934	29.5	Hb. 126 per cent. R. B. C. 6,430,000.
Average			23.30	857		Complete bile exclusion.

* 2–3 cc. of bile lost.

TABLE XLIII.

Known Diet With and Without Bile Exclusion.
Dog 18–54. Bile Fistula and Splenectomy.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Aug. 5	65	0.170	11.05	405	27.5	Absolute bile exclusion. Diet 280 gm. of cracker meal, 60 gm. of beef heart = 0.5 gm. of nitrogen and 100 calories per kilo.
" 6	62	0.181	11.23	412	27.8	
" 7	41	0.100	4.10	150	28.0	Binder chewed off night of the 6th.
" 8	47	0.182	8.56	314	27.5	
" 9	79	0.212	16.75	614	27.0	
Average.....			10.33	379		Complete bile exclusion.
Aug. 12	77±	0.224	17.25±	633	27.3	No bile exclusion. 5 hour collection.
" 13	51	0.140	7.14	262	27.3	
" 14	43	0.172	7.40	271	27.0	
" 15	57	0.115	6.56	241	27.0	
" 16	53	0.126	6.68	245	27.5	
Average.....			9.00	330		No bile exclusion.
Aug. 19	77	0.222	17.10	628	27.5	No bile exclusion.
" 20	76	0.167	12.68	465	27.3	Bile exclusion 12 hrs. previously.
" 21	71	0.187	13.26	486	27.5	Bile exclusion.
" 23	80	0.128	10.24	376	27.3	No bile exclusion.
Aug. 27	46				27.0	Absolute bile exclusion. Diet 417 gm. of beef heart and 100 gm. cracker meal = 1.0 gm. of nitrogen and 100 calories per kilo.
" 28	81	0.288	23.31	856	26.3	
" 29	75	0.311	23.32	856	26.5	
" 30	70	0.380	26.60	976	26.3	Hb. 110 per cent. R. B. C. 5,515,000.
Average.....			24.40	896		Complete bile exclusion.

TABLE XLIV.

Known Diet With and Without Bile Exclusion.
Dog 18–137. Bile Fistula and Splenectomy.

Date.	Volume.	Amino nitrogen.		Tauro-holic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
Aug. 5	53	0.313	16.58	608	23.0	Absolute bile exclusion. Diet 245 gm. of cracker meal, 45 gm. of beef heart = 0.5 gm. of nitrogen and 100 calories per kilo.
" 6	56	0.264	14.78	542	23.0	
" 7	38	0.365	13.86	509	23.3	
" 8	37	0.250	9.25	340	23.0	
" 9	34	0.198	6.73	247	23.0	
Average.....			12.25	450		Complete bile exclusion.
Aug. 12	56	0.196	10.96	402	22.8	No bile exclusion.
" 13	46	0.266	12.24	449	22.3	
" 14	40	0.214	8.56	314	22.0	
" 15	43	0.129	5.50	202	22.0	
" 16	44	0.198	8.71	320	22.0	
Average.....			9.20	338		No bile exclusion.
Aug. 19	50	0.404	20.20	742	22.0	3 hour collection.
" 20	57	0.292	16.65	611	22.3	Bile exclusion 12 hours previously.
" 21	68	0.188	12.78		22.3	Bile exclusion.
" 23	40	0.099	3.96	145	22.3	No bile exclusion.
Aug. 28					21.8	Absolute bile exclusion. Diet 327 gm. of beef heart and 100 gm. of cracker meal = 1.0 gm. of nitrogen and 100 calories per kilo.
" 29	50	0.335	16.75	611	22.0	
" 30	57	0.376	21.42	786	22.0	Hb. 120 per cent. R. B. C. 5,060,000.
Average.....			19.07	698		Complete bile exclusion.

Tables XLV and XLVI supply more data on bile exclusion and fasting excretion of bile acids. These tables are to be compared with Tables XXXII and XXXIV in Paper IV and the average of these last two tables is added to Tables XLV and XLVI now under consideration. Table XLV shows practically the same output of bile acids per 6 hours whether bile is excluded or not. This is in harmony with all the other experiments given in this and other papers as well as many experiments which are unpublished.

TABLE XLV.

Bile Exclusion—Fasting.
Dog 17–151. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
May 27	28	0.693	19.40	712	26.8	41.2	Hb. 120 per cent. R. B. C. 7,520,000.
" 28	24	0.541	12.98	476	23.8		
" 29	19	0.492	9.35	343	9.3	39.0	
" 30	20	0.558	11.20	411	9.0	38.0	
" 31	19	0.653	12.41	455	13.9	37.1	
June 1	23	0.694	15.96	586	10.0	36.5	
Average 6 days ..			13.55	497		Complete bile exclusion.	
Average 10 days ..			11.42	419		No bile exclusion. Table XXXII.	

Table XLVI presents a single contradiction to all the rest of our published data and taken by itself would seem to indicate that bile exclusion did actually diminish the bile acid output. Such contradictions are not unheard of in experimental work, but we believe this exception to the general rule is in reality explicable. This dog as stated above (Paper IV) is known to have a small opening from his biliary tract into the duodenum. At times he presents remarkable variations in the output of bile acids which stand in contrast to the other dogs whose biliary passages are absolutely separated from the duodenum. Because of this fact we attach little importance to observations on this dog

when the bile is concentrated and viscid, and still less importance to observations which differ from those made upon the other fistula dogs.

TABLE XLVI.

Bile Exclusion—Fasting.
Dog 15–22. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Bile pigments in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.				
1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*lbs.*	
May 27	27	0.905	24.30*	892*	3.7	34.5	Hb. 130 per cent. R. B. C. 6,540,000.
" 29	12	0.532	6.38	234	14.6	32.3	
" 30	21	0.435	9.14	335	18.3	31.5	
" 31	16	0.380	6.08	223	9.7	30.9	
June 1	12	0.562	6.74	247	14.6	30.4	
" 2	20	0.086	1.72	63	46.0	29.7	NH_3 determination repeated with same result.
" 3	16	0.510	8.16	299	24.5	29.4	
Average 6 days ...			6.37	234		Complete bile exclusion.	
Average 9 days ...			11.09	407		No bile exclusion. Table XXXIV.	

* Not included in average.

SUMMARY.

Complete exclusion of bile ingestion at all times does not modify the excretion of bile acids from day to day. This statement applies to the dogs used in our experiments and to the laboratory conditions under which all this work was done. It is not necessary therefore to take elaborate precautions to prevent any given dog from licking its fistula during afternoon and night resting periods.

These experiments confirm those tabulated in the preceding paper to show that an increase in the meat proteins in the diet will cause a considerable rise in output of taurocholic acid.

THE METABOLISM OF BILE ACIDS.

VI. ORIGIN OF TAUROCHOLIC ACID.

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Taurocholic acid can be easily separated into its two essential constituents, taurine and cholic acid. It has been shown (Paper III) that in the body the two constituent parts can be easily and rapidly united to form taurocholic acid. This holds true whether the two substances are given by mouth or whether cholic acid is given by mouth and taurine intravenously. It is clear that cholic acid and taurine have a strong physiological attraction in the normal body.

Taurine appears to be present in the normal body in a certain excess, judging from cholic acid feeding experiments. Taurine is more abundant in the body, at least more accessible for linkage with cholic acid, during full diet periods than during fasting periods (Paper III). We have also submitted evidence to show that taurine is derived at least in part from the cystine of the food. This is in harmony with the observations of von Bergman (2) and Wohlgemuth (14), who used somewhat inaccurate methods of analysis. It is highly probable that taurine may be derived from other substances under certain conditions but we have no direct proof concerning this point. When the metabolism of taurine is fully understood we may be nearer a complete understanding of the complex bile acid metabolism.

Cholic acid is a substance which defies the investigator, who up to the present time has learned little about its source or usefulness in the body. Cholic acid is of particular interest to us because its supply appears to be the normal limiting factor which determines the bile acid level of excretion. A sufficient supply of taurine in the body seems to obtain under all physiological conditions. There is sufficient evidence to show the important relationship that

exists between the basal nitrogen metabolism and bile acid metabolism. We believe that knowledge concerning the bile acid metabolism will be of value for a complete understanding of the fundamental body protein metabolism.

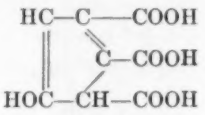
Cholic acid is somewhat complex and its accepted formula is given below. Because of its structural formula many hypotheses have been advanced concerning its origin in the body and its possible relationship to various drugs. Some positive experiments have been reported to indicate a relationship between cholic acid and cholesterol, but these data will not bear careful scrutiny. In the experiments given below we have tested a number of such hypotheses and without exception can report only negative results. It seemed to us of some importance to establish beyond a doubt that cholesterol and allied substances have no influence whatever on the cholic acid metabolism.

Through the work of von Bergman in 1904, and Wohlgemuth in the same year, it was clearly established that some of the taurine of the taurocholic acid came from cystine. Both used the sulfur method in their experiments. Friedman (4) in 1903 had shown that this change could be effected *in vitro*, but von Bergman was the first to show that when cystine is fed to a dog together with sodium cholate on a constant diet, there is a marked increase in the sulfur of the bile. There was a distinct but less striking increase when sodium cholate alone was given by mouth. There was no increase in bile sulfur when cystine alone was fed. Wohlgemuth followed the sulfur of the urine in cats before and after administering cystine. There was a large amount of neutral sulfur excreted, and, as there was no cystine in the urine, he concluded that all the cystine had been changed into taurine. This reasoning may be open to certain objections.

Gibson (5) gave three injections of bromobenzol to cats at 8 to 10 hour intervals to deflect the cystine from its usual cycle and found a marked decrease in the alcohol-soluble sulfur of the bile. He does not mention whether the cats were kept upon a uniform diet nor does he state their clinical condition after administering the drug. The cats were sacrificed 5 to 8 hours after the last injection. We know that this decrease in bile sulfur might have been caused by a diet low in meat protein nitrogen, by refusal of food, or by the intoxication from the bromobenzol.

Lifschütz (8) found that cholic acid gives the same color changes and spectrum as cholesterol or oxycholesterol when treated with acetic acid, benzoylsuperoxide, and sulfuric acid. Flury (3) states that the acids obtained by the oxidation of cholesterol may be placed in the pharmacological group of bile acids and saponins from their pharmacological behavior. He thinks that they may occur as intermediate steps in the formation of bile acids from cholesterol.

Excellent work has been reported by Schrötter, Weitzenböck, and Witt (11, 12). They found that cholesterol, cholic acid, oil of turpentine, and camphor all give the same compound, rhizocholic acid, when treated with concentrated sulfuric acid, mercury, and nitric acid. Rhizocholic acid is:



and was identified carefully in each case. From this fact they conclude that cholic acid and cholesterol belong to the terpene series.

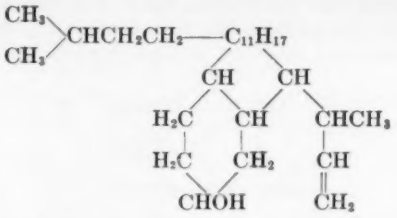
Pregl (10) states that cholic acid is closely related to turpentine and camphor and is a hexahydroxylbenzene. Moreschi (9) in 1913 attempted to show the relation of cholic acid to cholesterol by making a trichlor derivative from each. The cholic acid formed a compound with 11 atoms of chlorine, and cholesterol with 10 chlorine atoms.

Hammarsten (7) states that in certain animals (shark) the bile acids are replaced by scymnol sulfuric acid, made up of a substance he calls "scymnol" which is allied to cholic acid or cholesterol paired with sulfuric acid.

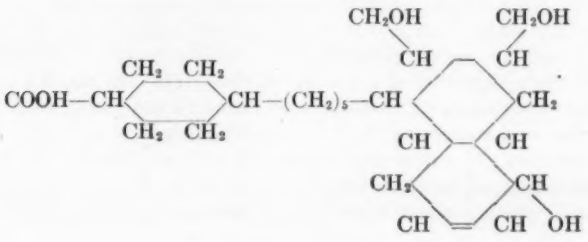
D'Amato (1) found that dogs fed on a lipoid diet gradually increased the excretion of both cholesterol and bile acids in the bile. The increase noted in bile acids is very small and within the limits of error of the method employed and surely within the physiological variations which are observed so frequently in routine collection periods.

Goodman (6) thought that the broken down red blood corpuscles might be available for the formation of bile constituents; *i.e.*, the cholesterol might be the mother substance of cholic acid. He used a method in which he weighed the cholic acid excreted. The diet consisted of dog biscuit (10 gm. of nitrogen per day), varied with red blood cells, egg white, coagulated horse serum, calves' brains, and cholic acid. The cholic acid caused a marked increase in the cholic acid content of the bile, but the bile cholesterol was unchanged. This cholic acid increase might have been due to cholic acid excreted as such, and not to taurocholic acid, for his method determines any cholic acid dissolved in the bile, whether united with taurine or not. The egg albumin increased the cholic acid but not the cholesterol. He used but one dog, and that dog lived only 4 weeks. For this reason and because of inaccuracies in his methods we do not attach much importance to his results. Because there is such a small amount of cholesterol present in the blood cells, Goodman says that in a human being there would be necessary about 60 per cent blood destruction per day, and even more in the dog, which of course is unthinkable. This theory at best could explain only a small part of the actual cholic acid production. Our experiments give no support to this suggestion.

According to Windaus (13), the formula for cholesterol is as follows:



Pregl gives the following formula for cholic acid:—



EXPERIMENTAL.

The experiments were conducted under conditions similar to those described above. The dogs were set up for ½ hour for free drainage of the bile fistula before the collections were started. Only a part of the experimental data is given, but it is uniform in character and the evidence all points the same way.

Table L presents two similar experiments in which cystine was given intravenously with no positive influence on the bile acid output, but with a decided effect upon the bile unhydrolyzed amino nitrogen fraction. This may indicate an excretion in the bile of cystine or taurine under these conditions. On the following day a similar injection of cystine followed by cholic acid by stomach resulted in a great increase in taurocholic acid excretion and a return of the unhydrolyzed amino nitrogen to normal. This gives evidence that under these conditions the body can change cystine rapidly to taurine, which is then available to combine with the cholic acid. It may be objected that on a mixed diet the feeding of cholic acid will increase the output of taurocholic acid because plenty of taurine is available from the food. Refer to Table XXIV, Paper III, where it is seen that the rise in tauro-

cholic acid does actually reach a higher level after administration of 4.0 gm. of cholic acid with a full mixed diet. It would be desirable to repeat these observations during a fasting period.

Taken together with the experiments of von Bergman and Wohlgemuth we feel reasonably secure in the statement that cystine can produce taurine under physiological conditions, and

TABLE L.

Cystine Intravenously Reacts with Cholic Acid.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
15–22	Nov. 25	53	0.227	12.04	442	33.3	Mixed diet.
	" 26	28	0.456*	12.75	468	33.0	0.7 gm. of cystine intravenously.
	" 27	51	0.746	38.05	1,395	32.5	0.7 gm. of cystine intravenously + 2.1 gm. of cholic acid by stomach.
17–34	Nov. 25	35	0.198	6.93	254	30.3	Mixed diet.
	" 26	40	0.356*	14.24	522	30.3	0.7 gm. of cystine intravenously. Vomited.
	" 27	76	0.453	34.45	1,264	30.3	0.7 gm. of cystine intravenously + 2.1 gm. of cholic acid by stomach. Vomited a little of cholic acid solution.

* The *unhydrolyzed* bile gave a high *unhydrolyzed* NH_2 showing the presence of an excess of cystine or taurine uncombined with cholic acid. The following day when cholic acid was given this *unhydrolyzed* NH_2 had returned to normal which would indicate that all the cystine had been synthesized into taurocholic acid.

this taurine is available to combine with an excess of cholic acid. This holds good apparently whether the cystine is fed by mouth (von Bergman) or given intravenously (Table L).

In comparing Table LI with Tables XXXI and XXXIII of Paper IV, one can see that these variations are within the normal fluctuations of these same dogs on a sugar diet.

Tables LI and LII are to be considered together and they furnish strong evidence that *cholesterol* alone or fed with taurine exerts no influence upon the excretion of taurocholic acid. This holds for periods of fasting as well as for periods of mixed diet. The amounts of cholesterol administered (3 to 4 gm.) are sufficient to convince any investigator that this substance does not play a part in bile acid metabolism. There is no immediate reaction nor any

TABLE LI.

Cholesterol Feeding—Sugar Diet—Bile Acids Unchanged.

Dog No.	Date.	Volume.	Amino nitrogen. In 1 cc. of bile.	Amino nitrogen. In 6 hours.	Taurocholic acid in 6 hours.	Pigments in 6 hours.	Urinary N in 24 hours.	Weight.	Remarks.
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*lbs.*	
17–151	Average 11 days.			8.05	295		3.45		Diet of 75 gm. of cane sugar, 100 gm. of glucose.
	Apr. 6	15	0.402	6.03	220		2.52	35.0	
	" 7	17	0.556	9.45	347	18.6	2.13	35.1	4 gm. of cholesterol.*
	" 8	17	0.416	7.08	260	14.2	2.57	34.4	
18–23	Average 11 days.			8.66	318		3.23		Diet of 75 gm. of cane sugar, 75 gm. of glucose.
	Apr. 6	37	0.171	6.32	232	14.2	3.30	25.8	
	" 7	31	0.269	9.34	343	13.0	2.58	25.7	4 gm. of cholesterol.*
	" 8	37	0.125	4.62	169	18.7	2.18	25.7	

* Given in gelatin capsule at beginning of 6 hour collection.

delayed effect to be observed. In Table LII the taurine was given to insure an excess of this substance in the body to combine with any amount of cholic acid available. Table LII illustrates the inhibition of bile flow due to sugar by mouth (Dog 15–22). Vomiting on certain occasions may be associated with a decreased flow of bile in these fistula dogs. The amount of ether used to dissolve the cholesterol (8 to 10 cc.) will have no influence upon the bile excretion.

Tables LIII and LIV are similar experiments which show that red blood cells by mouth have no effect upon the excretion of bile acids. The control periods of 5 days and the red blood cell feeding periods give figures which are practically identical. The

TABLE LII.

Taurine Plus Cholesterol Feeding—Bile Acids Unchanged.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
							Mixed diet.
18–93	Sept. 11	76	0.210	15.95	586	31.5	
	" 12	58	0.320	18.55	681	32.5	1 gm. of taurine,* 3 gm. of cholesterol. Some diarrhea. Intoxicated by ether.
	" 13	76	0.225	17.10	628	34.0	Hb. 126 per cent. R. B. C. 6,430,000.
15–22				6.40	235		Average 10 days fasting.
	June 6	3.6	0.642	2.31	84	28.2	100 gm. of cane sugar and 25 gm. of glucose after 10 days fasting with bile exclusion. Vomiting.
	" 7	10	0.616	6.16	226	27.4	2 gm. of taurine,* 3 gm. of cholesterol.
							Mixed diet.
18–54	Sept. 3	42	0.170	7.14	262	27.0	Hb. 110 per cent. R. B. C. 5,515,000.
	" 4	46	0.141	6.49	238	27.4	1 gm. of taurine* and 3 gm. of cholesterol. Intoxicated by ether.
	" 5	58	0.113	6.56	241	28.0	

* Taurine dissolved in water. Cholesterol dissolved in ether and given by stomach tube at beginning of 6 hour collection.

mixed diet gives considerable daily fluctuation, but the averages are nearly uniform. The addition of 10 gm. of dried red blood cells does not influence the curve of bile acid excretion. This amount of red cells contains an appreciable amount of cholesterol, but of course not comparable to the large amounts used in Tables

LI and LII. We have other experiments with brain feeding which show the same negative influence on bile acid excretion.

Table LV shows the negative reaction following the intravenous injection of laked red blood cells. This again illustrates how easily we may dissociate the excretion curves of bile pigments and

TABLE LIII.

Red Blood Cell Feeding—Bile Acids Unchanged.
Dog 18–23. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
July 15	56	0.272	15.23	559	31.8	Mixed diet. Absolute bile exclusion.
" 16	63	0.254	16.00	587	33.3	Hb. 115 per cent. R. B. C. 6,200,000.
" 17	67	0.297	19.90	731	32.5	
" 18	71	0.410	29.10	1,068	32.0	
" 19	62	0.324	20.18	741	32.3	
Average.....			20.00	734		
July 22	53	0.254	13.47	495	31.0	Mixed diet + 10 gm. of R. B. C.*
" 23	60	0.262	15.72	576	32.3	" " + 10 " " "
" 24	57	0.302	17.20	628	32.5	" " + 10 " " "
" 25	64	0.271	17.35	637	32.8	" " + 10 " " "
" 26	62	0.326	20.20	742		" " + 10 " " "
Average.....			16.80	617		

* Prepared by washing red blood cells in normal saline 3 times. The residual cells were dried by warm air and ground to a powder, 10 gm. of red cells made into an indefinite solution emulsion mixture, flavored with a little sugar and salt, made up to 400 cc. with water, and given by stomach tube.

bile acids. The intravenous injection of large amounts of laked red blood cells will cause a prompt and large increase in the output of bile pigments but no increase in bile acid excretion. There is no immediate and no delayed bile acid reaction which we have been able to observe. Some investigators have recorded a drop in bile acid excretion following intravenous injections of hemoglobin but our experiments are clear cut and negative in this respect. One

observes only the normal physiological fluctuations. A possible explanation of the observed depression of bile acid excretion is the febrile reaction which sometimes is observed following hemoglobin injections. No such reaction was observed in these experiments.

It is well to note that the whole laked cells were given—that is, stroma and hemoglobin. After the blood had been laked by dis-

TABLE LIV.

Red Blood Cell Feeding—Bile Acids Unchanged.
Dog 18–54. Bile Fistula and Splenectomy.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
July 22	37	0.268	9.91	363	28.0	Mixed diet. Bile exclusion.
" 23	41	0.213	8.71	319	28.3	
" 24	18	0.315	5.67	208	27.8	
" 25	33	0.299	9.87	362	27.5	
" 26	45	0.282	12.68	466		
Average.....			9.40	345		
July 30	65	0.125	8.13	299	28.0	Hb. 100 per cent. R. B. C. 5,075,000. Mixed diet + 10 gm. of R. B. C.*
" 31	48	0.237	11.36	417	28.3	" " + 10 " " "
Aug. 1	50	0.284	14.20	522	27.8	" " + 10 " " "
" 2	37	0.145	5.36	196	27.5	" " + 10 " " " Tube out. About 10 cc. lost.
Average.....			9.7	356		

* Red blood cells prepared as in Table LIII.

tilled water it was shaken thoroughly to insure a complete injection of stroma and cell fragments. It has been suggested repeatedly that the red cell stroma and perhaps the contained cholesterol were waste products which normally came to the liver to be transformed into bile acids and eliminated in the bile. This is an attractive hypothesis which is delightfully simple, but like many others it has no basis of experimental fact and should be put aside even if with regret.

Since Schrötter, Weitzenböck, and Witt were able to make rhizocholic acid from cholic acid, cholesterol, turpentine, or camphor, we thought it might be possible to produce cholic acid in the animal body by feeding either turpentine or camphor. Taurine was given on the last day in each experiment (Table LVI) so that any cholic acid which might be formed in the organism would unite with the taurine and be excreted in the bile as tauro-

TABLE LV.

Laked Red Cells Intravenously—Bile Acids Unchanged.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
18–23	July 29	66	0.262	17.28	637	30.5	Mixed diet.
	" 30	72	0.223	16.05	590	32.3	Laked blood.*
	" 31	58	0.349	20.25	744	32.3	Hb. 120 per cent. R. B. C. 6,375,000.
	Aug. 1	63	0.287	18.18	678	31.8	Laked blood.*
	" 2	51	0.413	21.03	772	32.3	
18–93	July 29	68	0.207	14.07	517	30.3	Mixed diet.
	" 30	66	0.241	15.90	584	31.8	Laked blood.*
	" 31	53	0.280	14.85	545	32.3	Hb. 135 per cent. R. B. C. 6,380,000.
	Aug. 1	70	0.229	16.02	588	32.3	Laked blood.*
	" 2	64	0.315	20.15	740	33.3	

* 100 cc. of sterile defibrinated normal blood, centrifuged, red cells washed and laked with water, made up to 100 cc., and kept on ice over night; rendered isotonic, warmed, shaken, and given intravenously by hypodermic needle 2 hours after start of collection.

cholic acid. This was probably an unnecessary precaution when we recall the great output of taurocholic acid which follows a cholic acid feeding during periods of mixed diet. This indicates an abundant source of taurine in the body during periods of liberal feeding, more than enough to combine with any expected excess of cholic acid.

Careful scrutiny of Table LVI reveals no fluctuations other than those observed in control periods, whether terpene hydrate alone or combined with taurine was given by stomach. There is no

TABLE LVI.

Terpene Hydrate With and Without Taurine by Mouth.

Dog No.	Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
			In 1 cc. of bile.	In 6 hours.			
	1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
							Mixed diet.
15–22	Sept. 3	22	1.150	25.30	929	33.3	Hb. 150 per cent. R. B. C. 7,455,000.
	" 4	47	0.214	10.05	369	34.5	1 gm. of taurine, 1 gm. of terpene hydrate.*
	" 5	45	0.381	17.15	630	33.5	1 gm. of taurine, 1 gm. of terpene hydrate.*
	" 6	38	0.388	14.73	541	32.8	
							Mixed diet.
18–23	Aug. 27	57	0.261	14.86	545	30.8	
	" 28	57	0.343	19.55	718	31.8	0.25 gm. of terpene hydrate.*
	" 29	70	0.309	21.63	794	33.3	0.5 gm. of terpene hydrate.* 0.5 gm. of taurine.
	" 30	53	0.434	23.00	844	33.3	
							Mixed diet.
18–54	Sept. 10	61	0.112	6.84	751	28.8	
	" 11	82	0.238	19.50	716	29.0	
	" 12	81	0.186	15.05	552	28.5	1 gm. of terpene hydrate,* 1 gm. of taurine by stomach tube. Diarrhea.
	" 13	47	0.155	7.29	257	27.8	
							Mixed diet.
17–181	Sept. 10	49	0.292	14.30	525	25.0	
	" 11	42	0.280	11.75	431	25.3	
	" 12	67	0.267	17.85	655	26.0	1 gm. of taurine, 1 gm. of terpene hydrate.* Diarrhea.
	" 13	42	0.324	13.60	499	25.0	
							Mixed diet.
18–93	Sept. 3	64	0.285	18.25	670	30.5	Hb. 126 per cent. R. B. C. 6,430,000.
	" 4	44	0.073	3.94	144	33.5	1 gm. of taurine and 1 gm. of terpene hydrate.* Diuresis.
	" 5	54	0.183	9.89	363	32.5	1 gm. of taurine, 1 gm. of terpene hydrate.*
	" 6	64	0.291	18.61	684		

* Terpene hydrate dissolved in a little dilute alcohol; taurine dissolved in water, given by stomach tube at beginning of 6 hour collection.

cholagogue action and no change in the bile concentration and total amounts of bile acids.

Table LVII shows the results of administration of camphor by mouth. Repeated doses of spirits of camphor have no effect upon the bile acid excretion. Similar observations have been made on

TABLE LVII.

Camphor Feeding With and Without Taurine by Mouth.
Dog 15–22. Simple Bile Fistula.

Date.	Volume.	Amino nitrogen.		Taurocholic acid in 6 hours.	Weight.	Remarks.
		In 1 cc. of bile.	In 6 hours.			
1918	*cc.*	*mg.*	*mg.*	*mg.*	*lbs.*	
July 29	48	0.290	13.92	511	33.5	Mixed diet.
" 30	29	0.155	4.49	165	34.3	0.2 gm. of camphor* by stomach tube. Hb. 135 per cent. R. B. C. 6,430,000.
" 31	35	0.292	10.22	375	34.3	0.4 gm. of camphor by stomach tube.
Aug. 1	38	0.430	16.34	600	33.5	0.6 gm. of camphor by stomach tube. Considerable salivation.
" 2	14	1.030	14.41	529	33.5	
" 5	47				33.8	0.4 gm. of camphor by stomach tube.
" 6					35.0	0.4 gm. of camphor by stomach tube.
" 7	53	0.269	14.25	523	34.3	0.4 gm. of camphor by stomach tube.
" 8	63	0.222	14.00	514	33.3	0.4 gm. of camphor and 1 gm. taurine by stomach tube.
" 9	40	0.439	17.56	644	34.0	0.4 gm. of camphor by stomach tube.

* Camphor given as a 10 per cent solution—spirits of camphor. Diuresis was uniformly noted.

other dogs. Obviously there is no immediate and no delayed reaction which can be attributed to the action of this drug.

It is evident that neither camphor nor terpene hydrate have any effect on the excretion of taurocholic acid. Cholesterol, turpentine, cholic acid, and camphor may all belong to the same group chemically, but physiologically they certainly are not closely related, at least as regards bile acid metabolism.

SUMMARY.

Taurine as found in the body is derived in all probability from the cystine of the food or body protein. Taurine appears to be present in excess of the amount needed to combine with the cholic acid of normal metabolism.

Cholic acid is the limiting factor which determines the level of bile acid excretion in the bile. The origin and fate of cholic acid have not been satisfactorily determined.

Cholesterol fed alone or combined with taurine causes no change in the excretion of bile acids. This gives no evidence of any physiological relationship between cholesterol and cholic acid.

Red blood cells fed by mouth or hemolyzed and injected intravenously have no influence upon the level of bile acid excretion.

Terpene hydrate and camphor fed alone or combined with taurine do not influence the curve of bile acid excretion.

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